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Award Number: W81XWH-08-1-0053

TITLE: Mechanisms of KAI1/CD82-Induced Prostate Cancer Metastasis

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REPORT DATE: August 2011

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE		*Form Approved* OMB No. 0704-0188

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1. REPORT DATE *(DD-MM-YYYY)* 01-08-2011	2. REPORT TYPE Final	3. DATES COVERED *(From - To)* 1 Feb 2008 - 31 Jul 2011
4. TITLE AND SUBTITLE Mechanisms of KAI1/CD82-Induced Prostate Cancer Metastasis		5a. CONTRACT NUMBER
		5b. GRANT NUMBER W81XWH-08-1-0053
		5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S) Cynthia Miranti E-Mail: cindy.miranti@vai.org		5d. PROJECT NUMBER
		5e. TASK NUMBER
		5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Van Andel Institute Grand Rapids, MI 49503		8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSOR/MONITOR'S ACRONYM(S)
		11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Metastatic prostate cancer kills over 28,000 American men every year. The mechanisms that drive metastasis are poorly understood. We investigated the hypothesis that loss of the metastasis suppressor gene, CD82/KAI1 in primary prostate tumors of genetically engineered mice would be sufficient to induce metastasis. While CD82 was sufficient to suppress metastasis of metastatic tumor cells lines and did so by repressing c-Met, the reverse was not true, i.e. loss of CD82 was not sufficient to induce metastasis. This was the case in at least two genetically engineered prostate cancer models. Ongoing studies in different models may yet prove otherwise. Our other hypothesis, that CD82 was suppressing c-Met and invasion through integrins, was proven invalid in these studies. Other tetraspanins that CD82 associates with, i.e. CD9 and CD151 were required to suppress c-Met, but not invasion. Thus, these two events must be occurring via different mechanisms. Nonetheless, we are finding several interesting phenotypes in the CD82 null mice which will shed additional light on CD82 function overall and be useful in developing new hypotheses about how CD82 functions as a metastasis suppressor.

15. SUBJECT TERMS

cell biology, integrins, KAI1/CD82, tetraspanins, metastasis, c-Met, HGF, mouse models

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U	UU	40	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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INTRODUCTION

Prostate cancer is the most frequently diagnosed cancer and the third leading cause of cancer deaths in men in the United States.[1] Death is due to invasion and metastasis beyond the prostate gland, primarily into the bone and occasionally to other peripheral organs. Our basic understanding of how prostate cancer metastasis develops is limited. The recent identification of genes, whose expression suppresses metastasis but not growth in xenograft models, has provided a potential avenue for better understanding the metastatic process.[2] **The overall objective of this proposal was to determine how loss of the metastasis suppressor, KAI1/CD82, promotes the development of metastatic prostate cancer.**

Elevated expression of two integrins $\alpha6\beta1$ and $\alpha3\beta1$ is highly correlative with the invasive and metastatic phenotype of prostate cancer. It has been proposed that migration of tumor cells on laminin-enriched nerve fibers via $\alpha6\beta1$ and $\alpha3\beta1$ integrins facilitates prostate cancer spread.[3] The metastasis suppressor KAI1/CD82 is known to associate with $\alpha6\beta1$ and $\alpha3\beta1$ integrin laminin receptors. We previously demonstrated that adhesion of metastatic prostate cancer cells to laminin induces activation of the metastasis associated receptor tyrosine kinase c-Met, and that re-expression of KAI1/CD82 suppresses both laminin- and HGF-induced c-Met activation.[4] c-Met is up-regulated in all metastatic prostate cancers and is a physiological mediator of cell migration and invasion.[5] **Thus we hypothesized that loss of CD82/KAI1 expression in primary prostate cancer results in enhanced activation of c-Met via both its ligand HGF and laminin integrins, which influences downstream signals required to promote metastasis.**

BODY

Summary of Aim 1. The goal of aim 1 was to identify the mechanism by which CD82 regulates c-Met activity. Our *working hypothesis* was that CD82 negatively regulates c-Met activation through CD82-specific association with integrins and other tetraspanin molecules. Our **first task** was to determine the region on CD82 that interacts with integrins. To accomplish this we generated a CD82 mutant with a deletion in part of the extracellular domain (EC2) that was proposed to no longer interact with integrins.[6] In addition, we obtained a CD82 mutant that is defective in palmitoylation and unable to bind other tetraspanins.[7]

Initially we generated a few cell lines expressing the palmitoylation mutant, but soon discovered this protein was not stable. Upon sequencing the construct that was provided to us from another lab,[8] we discovered several mutations within it which were unrelated to the palmitoylation sites. We regenerated this mutant in our own lab, but were still not able to establish stable cell lines expressing it. In an attempt to overcome this, we generated several mutants in which predicted ubiquitination sites within the two cytoplasmic tails were mutated in the hopes of increasing protein stability. We were able to generate these cell lines (Fig 1A), and found that mutation of Lys7/Lys10 in the N-terminal tail significantly stabilized CD82 expression and were the sites of ubiquitination (Fig 1B). GP78 is the E3 ligase that ubiquitinates CD82.[9] Using a CD82 expressing clone that lost CD82, we found that knocking-down gp78 restored CD82 expression in these cells (Fig 1C). This mutant had an increased ability to suppress Matrigel invasion compared to wild type CD82 (Fig 1D). Thus, we can use either of these strategies to stabilize expression of other CD82 mutants.

Figure 1: A) Levels of CD82 expression in DU145 cells stably infected with retroviruses expressing vector (pLPCX), wild-type (WT) or different lysine (K) to arginine (R) mutants in different combinations aimed at 4 sites in the cytoplasmic tails predicted to be ubiquitination sites, L7, L10, L263, and L266. Tubulin is the loading control. **B)** The predicted ubiquitin defective CD82 mutant cell lines (KR) were transfected with HA-tagged ubiquitin and the level of HA-ubiquitin (HA-Ubn) in CD82 immunoprecipitates was measured by immunoblotting with HA antibody. Only the L7/10R mutant had reduced ubiquitin incorporation. **C)** A PC3 cell line in which CD82 was originally engineered to be stably expressed and then lost CD82 expression upon subsequent passage. Treatment of that cell line with gp78

shRNA restored CD82 expression, but had no effect on CD9. **D)** DU145 cells expressing wild type (WT) CD82 or the KR mutants were tested for their ability to invade Matrigel in the presence of 100ng/ml HGF in vitro. At this concentration of HGF, wild-type CD82 is a weak suppressor of invasion. However, the L7/10R mutant suppressed invasion better than the wild-type or other mutants in which ubiquitination was not impaired.

As reported last year, we successfully generated a few stable PC3 cell lines expressing a C-terminally HA-tagged extracellular deletion mutant and its wild-type tagged counterpart. As previously reported, using this mutant we were able to demonstrate 1) in CD82 expressing cells, there is an $\alpha6\beta1$ integrin complex containing CD82 and CD151, and the complex between $\alpha3\beta1$ and CD9 is dissociated; 2) CD82 prevents HGF-induced changes in CD9 association with integrin/tetraspanin complexes; and 3) c-Met was present only in the CD82/CD151/$\alpha6\beta1$ complex. Thus, CD82 seems to favor the formation of a complex between $\alpha6\beta1$/CD151/c-Met, at the expense of breaking apart a complex between CD9/CD151/$\alpha3\beta1$ integrin.

Our **second task** was to identify the region on CD82 that is responsible for suppressing c-Met activity. The EC2 domain deletion mutant failed to suppress c-Met activity as reported last year. Thus, the extracellular domain of CD82 is required for its ability to suppress c-Met activity.

Our **third task** is to determine if CD82 interaction with integrins/tetraspanins is responsible for suppressing c-Met activity. As reported last year, we found that both CD9 and CD151 are required for CD82 to suppress c-Met. Neither integrin $\alpha3$ nor $\alpha6$ is required for c-Met activation, nor are they required for CD82 to suppress c-Met. The ability of CD82 to suppress invasion was not reversed by loss of CD151, $\alpha3$ or $\alpha6$ integrin. These data suggest that the effect of CD82 on c-Met suppression mediated through CD151 and CD9 are not likely to be responsible for the effect of CD82 on invasion.

In summary, we have found a disconnect between the mechanisms by which CD82 suppresses c-Met versus how it suppresses invasion. Tetraspanins CD151 and CD9, but not the integrins, are required for c-Met suppression. None are required to suppress invasion. On the other hand, CD151 and $\alpha3\beta1$ and $\alpha6\beta1$ all contribute to invasion and their loss cooperates with CD82 to suppress invasion.

Summary of Aim 2. The goal of aim 2 was to determine how loss of CD82 leads to metastatic prostate cancer. Our *working hypothesis* was that CD82 loss *in vivo* results in increased c-Met signaling, both of which are required for the development of metastatic disease. Our **first task** was to determine if CD82 expression inhibits metastasis in HGF transgenic mice. We previously reported that HGF/c-Met dependent metastasis of DU145 cells was inhibited by expression of wild-type CD82, and is accompanied by a loss in c-Met activation *in vivo.*

We wanted to test the CD82 mutants in the same assay, but because of the unstable nature of those cell lines, we were never able to do this. But, now that we have a mutant with enhanced stability (i.e. Lys7/Lys10), we will engineer the extracellular domain mutation and the palmitoylation mutation into it and then test them in vivo.
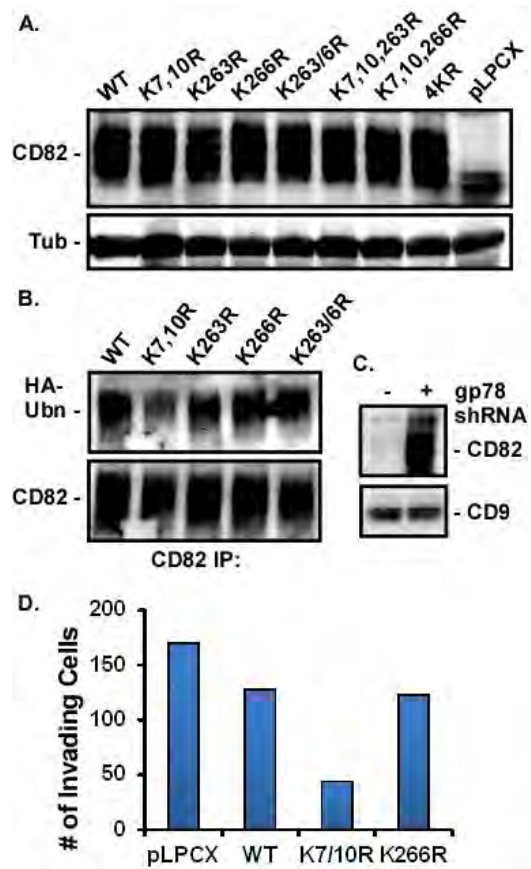
Our **second task** was to determine if loss of CD82 expression in mice genetically manipulated to produce prostate tumors is required for the development of metastatic disease. CD82 floxed mice were crossed to CMV-CRE mice to generate a complete knock-out of CD82, or to Probasin-Cre mice to generate loss of CD82 only in prostate epithelial cells. As previously reported, loss of CD82 in the prostate has no effect on normal prostate physiology.

The CD82/Probasin-Cre mice were crossed to PTEN floxed mice to generate combined loss of both CD82 and PTEN in the prostate. Loss of CD82 in the context of PTEN loss had no effect on either tumor growth, number of tumors, or metastatic potential. The CD82/Probasin-Cre mice were also crossed to APC floxed mice[10] to generate combined loss of both APC and CD82. As was seen for PTEN, loss of CD82 did not affect tumor growth, number of tumors or produce metastases.

To assess whether genetic background could impact metastasis, we backcrossed all three strains, CD82$^{-/-}$, Probasin-Cre, and Pten$^{flox/flox}$ mice into FVB/N which are generally more susceptible to tumorigenesis. We crossed the FVB/N strains together and the analysis is still ongoing. It is possible that loss of more than one metastasis suppressor is required for metastasis. Therefore, we obtained RKIP null mice. RKIP, like CD82, is a metastasis suppressor gene; however, it functions differently than CD82.[11] RKIP suppresses the MAPK and Myc pathways, while CD82 suppresses Src, PKCs and RTKs. Thus, we reasoned that activation of several signaling pathways maybe required for metastasis. We had to rederive the RKIP line in our facility, which was done, and we now have RKIP/CD82 compound homozygotes. These will be crossed into the APC floxed/PB-Cre mice. Finally, to test whether combined loss of CD82 and APC in earlier progenitor cells of the prostate is required for metastasis, we crossed the CD82/APC floxed mice to the newly developed Nkx3.1-ER Cre mouse. We have started inducing Cre with tamoxifen and are currently monitoring tumor formation and metastasis.

In summary, we have shown that CD82 is sufficient to suppress metastasis and c-Met activation in vivo in metastatic cells. Our in vitro data suggests this is mediated by the extracellular domain of CD82. However, loss of CD82 is not sufficient, at least in the two models analyzed thus far (i.e. PTEN or APC), to generate metastases. Ongoing studies will determine if genetic background, progenitor cell, or loss of a second metastasis gene will be sufficient to generate metastatic tumors.

KEY RESEARCH ACCOMPLISHMENTS

1. Generated stable transfectants of PC3 and DU145 cells expressing a deletion mutant of CD82. However, the DU145 cell mutants were unstable.
2. Generated stable transfectants of DU145 cells expressing ubiquitination defective mutants of CD82 and demonstrated they have increased invasion and c-Met suppressive activities.
3. Demonstrated that the EC2 domain of CD82 is required for CD82-mediated suppression of c-Met activity and invasion.
4. Demonstrated that CD82 preferentially associates with a CD151/α6α1/c-Met complex and dissociates CD151 and CD9 from their association with α3β1. CD82 also independently associates with CD9.
5. Demonstrated that CD9 and CD151, but not integrins, are required for CD82-mediated suppression of c-Met activity.
6. Tetraspanins CD9 and CD151 nor integrins α3 and α6 are required for CD82 suppression of invasion, but rather CD151, α3, and α6 loss cooperate with CD82 to suppress invasion.
7. CD82 suppresses metastasis and c-Met activation in vivo.

8. Failed to generate metastatic lesions in mice lacking CD82 in prostate epithelial cells in the context of Probasin-Cre/Pten -/- or Probasin-Cre/APC-/- mice in a mixed genetic background.
9. Generated FVB/N strains of CD82-/-, Probasin-Cre, and Pten$^{flox/flox}$ mice, crossed them together and are analyzing the outcome.
10. Generated CD82/APC floxed x Nkx3.1-ER Cre mice and are analyzing them.
11. Generated CD82/RKIP double null mice and preparing to cross them to APC/Pb-Cre

REPORTABLE OUTCOMES

The following items have been generated due to the research carried out during the three year award period.

1. We have published an invited review paper on the role of CD82 in metastasis. A copy is in the appendix.

> **Miranti, C.K.** 2009. Controlling cell surface dynamics and signaling: how CD82/KAI1 suppresses metastasis. **Cellular Signaling** 21:196–211.

2. Two abstracts were delivered as oral presentations at scientific meetings. The abstracts are in the appendix.

> Presented by Dr. Miranti:
> **Miranti, C.K.** 2008. CD82 Suppresses Metastasis via Inhibition of c-Met. **FASEB: Signal Transduction through Tetraspanins and Other Multi-Protein Cell Surface Complexes**, New Haven, Connecticut, June 22-29.
>
> Saari KM, Edick MJ, Vyas JM, Kasperkovitz PV, Risinger JI, Barrett JC and **Miranti CK**. 2010. Phenotypic Analysis of CD82 Null Mice. **4th European Conference on Tetraspanins**. University of Birmingham, England, Sept 8-10.

3. Eleven posters were presented at scientific meetings. The abstracts are in the appendix.

> Presented by Dr. Miranti:
> Saari KM, Spotts S, Rajah G, Tesfay L, Schulz VS and **Miranti CK** (2008) CD82/KAI1 Suppresses Prostate Cancer Metastasis via Inhibition of the receptor tyrosine kinase Met. **Metastasis - AACR/MRS Joint Meeting**, Vancouver, BC, Aug 3-7.
>
> Saari KM, Spotts S, Rajah G, Tesfay L, Schulz VS and **Miranti CK** (2009) Mechanism of Metastasis Suppression by KAI1/CD82: Integrins, Tetraspanins, and Suppression of c-Met Activation. **Beatson International Cancer Conference: Microenvironment, Motility, and Metastasis**, Glasgow, Scotland, July 5-8.
>
> Park E and **Miranti CK** (2010) Regulation of E-Cadherin and Cell-Cell Adhesion by the Metastasis Suppressor Tetraspanin KAI1/CD82. **Metastasis and the Tumor Microenvironment (AACR/MRS Joint Meeting)**, Philadelphia, PA, Sept 12-15.
>
> Saari KM, Spotts SM, Park ER, Rajah G, Schulz VS and **Miranti CK** (2011) CD82/KAI1 Suppresses Prostate Cancer Metastasis via Inhibition of the Receptor Tyrosine Kinase Met. **Innovative Minds in Prostate Cancer Today – IMPaCT Meeting**. Orlando, FL, Mar 9-12.

Presented by Ms. Saari:

Saari KM, Edick MJ, Sian KR and Miranti CK (2009) Development of a Total Kai1/CD82 Murine Knockout. **9th Annual Symposium Michigan Prostate Research Colloquium**, Wayne State University, Detroit, MI, May 30.

Saari KM, Edick MJ, Sian KR and Miranti CK (2010) Development and Investigation of a Total KA11/CD82 Murine Knockout. **10th Annual Symposium Michigan Urological Oncology Research Colloquium**, University of Michigan, Ann Arbor, MI, June 11-12.

Presented by Dr. Park:

Park E and Miranti CK (2009) Tetraspanin Kai1/CD82 Regulation of Cell-Cell adhesion: A Mechanism of Metastasis Suppression? **9th Annual Symposium Michigan Prostate Research Colloquium**, Wayne State University, Detroit, MI, May 30.

Park E and Miranti CK (2009) Tetraspanin KAI1/CD82 Regulation of Cell-Cell Adhesion: A Mechanism of Metastasis Suppression? **Gordon Research Conference: Cell Contact and Adhesion**, Waterville Valley, NH, June 28-July 3.

Park E and Miranti CK (2010) Regulation of E-cadherin and Cell-Cell Adhesion by the Tetraspanin CD82. **10th Annual Symposium Michigan Urological Oncology Research Colloquium**, University of Michigan, Ann Arbor, MI, June 11-12.

Park E and Miranti CK (2010) CD82 Regulation of E-Cadherin: A Mechanism of Metastasis Suppression. **Society for Basic Urological Research Annual Meeting**, Atlanta, GA, Nov 11-14.

Park E and Miranti CK (2011) CD82 Regulation of E-cadherin: A Mechanism of Metastasis Suppression. **FASEB: Membrane Organization by Molecular Scaffolds**, Saxtons River, VT, July 17-22.

3. We generated stable PC3 cell lines expressing extracellular mutants of CD82 and DU145 cells expressing ubiquitination-defective mutants. We are the first to do this and these will be useful for others interested in studying CD82 function.

4. We developed an HGF/c-Met-dependent metastasis model using DU145 cells and HGF/SCID mice. This model will be valuable for assessing c-Met-dependent prostate tumors and metastasis, and potentially as a preclinical drug screening model.

5. We generated the first conditional CD82 knock-out mouse. This KO is available in mixed, c57BL6 or FVB/N backgrounds. This model will be useful for analyzing the role of CD82 in many biological/organ processes, useful for distinguishing cell origin of specific phenotypes, and can be used to assess the role of CD82 suppression of metastasis in any primary cancer model.

6. We generated the following genetic mouse strains, all of which are useful to the prostate cancer research community:

$CD82^{flox/flox}/PTEN^{flox/flox}$/Probasin-Cre in both mixed and FVB/N backgrounds
$CD82^{flox/flox}/APC^{flox/flox}$/Probasin-Cre
$CD82^{flox/flox}/APC^{flox/flox}$/Nkx3.1-ER Cre
$CD82$/RKIP null x $APC^{flox/flox}$/Probasin-Cre mice

CONCLUSIONS

Prior to our studies the role of CD82 loss in regulating prostate tumor metastasis was unknown. We demonstrated that in tumor cells where c-Met expression is responsible for enhancing migration and invasion *in vitro*, re-expression of CD82 suppresses c-Met function. We also showed this to be true *in vivo*. We generated an extracellular domain deletion mutant of CD82 that no longer suppresses c-Met and no longer suppresses invasion, indicating this domain is important for CD82's activity. Our original hypothesis that CD82 was suppressing c-Met and invasion through integrins was proven invalid in these studies. Other tetraspanins that CD82 associates with, i.e. CD9 and CD151 are required for c-Met suppression, but not invasion in vitro. Thus, these two events must be occurring via different mechanisms. We generated ubiquitination-deficient mutants that increase the stabilization of CD82, which will be necessary for assessing the functions other CD82 mutants. While CD82 is sufficient to suppress metastasis of metastatic tumor cells lines, the reverse is not true, i.e. loss of CD82 was not sufficient to induce metastasis. This is the case in at least two genetically engineered prostate cancer models. Ongoing studies in different models may yet prove otherwise. We are finding several interesting phenotypes in the CD82 null mice which will shed additional light on CD82 function overall and could be useful in developing new hypotheses about how CD82 functions as a metastasis suppressor.

So What: Our findings have broad implications for the control of metastatic cancer. CD82 loss has been reported in many types of cancers. Likewise, c-Met over expression, mutation, or activation has also been reported for a wide range of cancers and its aberrant activity correlates with the development of metastasis.[12-14] We proposed that loss of CD82 may be required for the development of metastasis, by removing a control point for c-Met signaling. Our in vivo studies strongly suggest this is true; however, the mechanism by which this occurs is still not clear, though it involves other tetraspanins like CD151 or CD9. Interestingly, two other tetraspanins, CD151 and CO-029, appear to behave opposite to CD82, in that their levels of expression and activity are elevated in tumors.[15,16] Loss of CD82 appears to act, in part, by enhancing the activity of other tetraspanins to drive metastasis. We also learned that loss of CD82 is not likely to be sufficient to generate prostate cancer metastasis; thus, there are other components involved in metastasis development. Our ongoing studies with another metastasis suppressor, RKIP, may shed more light on how prostate cancer metastasis develops.

PERSONNEL SUPPORTED BY GRANT

Dr. Cynthia Miranti	20% effort	02/01/08 - 07/31/11
Kristen Saari	100% effort	03/01/08 - 02/05/11
Susan Spotts	50% effort	02/01/08 - 06/30/09
Dr. Electa Park	50% effort	06/01/09 - 06/30/11

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APPENDIX - MEETING ABSTRACTS

Oral Presentations:

Miranti, C.K. 2008. CD82 Suppresses Metastasis via Inhibition of c-Met. **FASEB: Signal Transduction Through Tetraspanins and Other Multi-Protein Cell Surface Complexes**, New Haven, Connecticut, June 22-29.

CD82 Suppresses Metastasis via Inhibition of c-Met
Cindy K. Miranti
Van Andel Research Institute, Grand Rapids, MI

Loss of CD82 expression in prostate cancer correlates with progression to metastasis. We have demonstrated that re-expression of CD82 at physiological levels in metastatic prostate tumor cell lines, suppresses integrin- or HGF-mediated activation of the receptor tyrosine kinase c-Met. Signaling through c-Met is required for cell migration and invasion in metastatic prostate tumor cells and is over expressed in all metastatic prostate cancers. We are investigating the role of CD82 in suppressing c-Met in vivo and determining the mechanism by which CD82 suppresses c-Met activity. Orthotopic injection of DU145 cells into SCID mice over expressing human-specific HGF results in 100% induction of prostate cancer metastases, compared to 0% metastases after injection into normal SCID mice. CD82 re-expression in DU145 cells suppresses prostate cancer metastasis by 95%, but has no effect on primary tumor growth. Activated c-Met could be detected in primary and metastatic tumors from HGF-SCID mice, while activated c-Met was not detected in primary tumors from CD82-expressing cells. We have successfully generated CD82 null mice, which appear normal and are fertile. The CD82 mice are being crossed with Pten conditional mice, which develop primary but not metastatic prostate cancer, to determine if loss of CD82 is sufficient to induce prostate cancer metastasis in Pten null mouse prostates. Co-immunoprecipitation experiments have failed to detect a direct interaction between CD82 and c-Met, thus the effect of CD82 on c-Met must be indirect. CD82 is known to associate with integrins and integrins can control c-Met activation. CD82 was found to associate with $\alpha3\beta1$ integrin and CD9 in prostate cells. siRNAs directed to CD9 blocked the ability of CD82 to suppress c-Met activation. A small deletion in the EC2 domain, but not inhibition of palmitoylation, generated a CD82 mutant that failed to inhibit c-Met activation. These data indicate that the extracellular domain of CD82 and CD9 are required to suppress c-Met activation.

Saari KM, Edick MJ, Vyas JM, Kasperkovitz PV, Risinger JI, Barrett JC and **Miranti CK**. 2010. Phenotypic Analysis of CD82 Null Mice. **4th European Conference on Tetraspanins**. University of Birmingham, England, Sept 8-10.

Phenotypic Analysis of CD82 Null Mice

Saari KM[1], Edick MJ[1], Vyas JM[2], Kasperkovitz PV[2] Risinger JI[3], Barrett JC[4] and Miranti CK[1]

[1]Laboratory of Integrin Signaling, Van Andel Research Institute, Grand Rapids, MI
[2]Department of Medicine, Infectious Disease, Massachusetts General Hospital, Boston, MA
[3]Department of Obstetrics, Gynecology and Reproductive Biology, College of Human Sciences, Michigan State University, Grand Rapids, MI
[4]Novartis Institutes for BioMedical Research, Cambridge, MA

Mice harboring homozygous floxed alleles of CD82 were generated to gain a better understanding of CD82 function in mammalian cells. CMV-Cre driven recombination and deletion of either exons 2+3 or exons 4+5 generates mice that are completely viable and fertile in mixed 129/BL6 or BL6 backgrounds. CD82 protein or mRNA is not detectable in tissues from the mice. CD82 loss was not accompanied by a compensatory increase in tetraspanins CD9, CD81, or CD151. No gross anatomical or histological abnormalities are readily apparent. Highest levels of CD82 expression were detected in tissues taken from the kidney, liver, and spleen. In addition, CD82 staining was prominent in endothelial vessels. Following a more detailed analysis and pathogenic challenge, abnormalities were detected in platelets, antigen presentation, angiogenesis, and kidney function.

In a mixed 129/BL6 background recovery of retinal vasculature was normal following oxygen-induced retinopathy. However, in bFGF Matrigel plug assays, there was a 22% increase in Matrigel plug weight and vasculature in the KO mice compared to WT controls. The enhanced angiogenesis seen in CD82 KO mice phenocopies what is observed in endothelial-specific integrin α3 null mice. In tail clipping assays, blood clotting occurred significantly faster in KO mice compared to WT controls and KO mice displayed a 30% increase in platelet numbers. CD82 was found to be expressed on the cell surface of both human and mouse platelets. CD82 surface expression was independent of platelet activation state. A defect in innate TLR9 signaling was observed in CD82 null antigen presenting cells. CpG failed to induce TLR9-dependent, TNF-α secretion from bone marrow-derived dendritic cells. Restoration of CD82 expression in these cells permitted TLR9-dependent, TNF-α secretion. CD82 is recruited to the phagosomal surface prior to CD63 and is coincident to class II MHC arrival. $CD82^{-/-}$ DCs loaded with ovalbumin failed to stimulate Ova-specific T-cells as well as wild-type dendritic cells. Increased urine and fecal water content was observed in CD82 null mice, as well as the presence of clear crystals within the collected urine. Transmission EM revealed an increased number of lipid whorls in tubular epithelial cells, reminiscent of structures associated with lysosomal storage diseases. Mice are currently being bred onto an FVB/N background. To date, crossing CD82 KO mice with PTEN or APC prostate-specific deleted mice has failed to generate tumor metastases.

Loss of CD82 has subtle pathological effects in cell types in which loss of other tetraspanins also generates defects. The defects seen in platelets and endothelial cells are opposite to those observed in CD151 KO mice, while the defects in TLR9 and the kidney mirror those seen in CD63 null mice.

Poster Presentations:

Saari KM, Spotts S, Rajah G, Tesfay L, Schulz VS and **Miranti CK** (2008) CD82/KAI1
    Suppresses Prostate Cancer Metastasis via Inhibition of the receptor tyrosine kinase
    Met. **Metastasis - AACR/MRS Joint Meeting**, Vancouver, BC, Aug 3-7.

**CD82/KAI1 Suppresses Prostate Cancer Metastasis via Inhibition of the receptor tyrosine
kinase Met**
Kristen Saari, Susan Spotts, Gary Rajah, Lia Tesfay, Veronique Schulz, and <u>Cindy K. Miranti</u>
Van Andel Research Institute, Grand Rapids, MI

Expression of the tetraspanin protein, CD82/KAI1, is lost in metastatic tumors from prostate
cancer patients and its loss correlates with poor prognosis. The receptor tyrosine kinase Met is
over expressed in metastatic prostate cancers and promotes cell migration and invasion. We
demonstrated that re-expression of CD82 at physiological levels in metastatic prostate tumor
cell lines, suppresses integrin- or HGF-mediated activation of Met. We are investigating the role
of CD82 in suppressing Met *in vivo* and determining the mechanism by which CD82 suppresses
Met activity. CD82 and its related family members, CD9 and CD151, are known to associate
with integrins and integrin $\alpha3\beta1$ in prostate cancer cells is required for efficient Met activation.
Furthermore, siRNAs directed to CD9/D151 blocked the ability of CD82 to suppress Met
activation, indicating CD82 association with CD9/CD151 is required for its ability to suppress
Met. In addition, a small deletion in the extracellular domain of CD82 generated a mutant that
failed to inhibit Met activation. Together these data indicate that the extracellular domain of
CD82 and its interaction with CD9/CD151 are required to suppress Met activation. We are
currently determining whether the association of integrin $\alpha3\beta1$ with the tetraspanins is required
to suppress Met activation. Orthotopic injection of DU145 cells into SCID mice over expressing
human-specific HGF results in 100% induction of prostate cancer metastases, compared to no
metastases after injection into normal SCID mice. CD82 re-expression in DU145 cells
suppresses prostate cancer metastasis by 95%, but has no effect on primary tumor growth.
Activated Met could be detected in primary and metastatic tumors from HGF-SCID mice, while
activated Met was not detected in primary tumors from CD82-expressing cells. We are currently
determining whether the CD82 deletion mutant is incapable of suppressing metastasis *in vivo*.
In addition, we generated CD82 conditional null mice which were then crossed to the prostate-
specific Pten or APC conditional null mice, to test the hypothesis that loss of CD82 would be
sufficient to generate metastatic lesions from the primary prostate tumors that developed in
these mice. Unfortunately, none of the mice developed metastasis. Thus, while loss of CD82
may contribute to metastasis, it alone is not sufficient. We are currently exploring the
contribution of other tumor metastasis suppressors in combination with CD82 to generate
prostate cancer metastases.

Saari KM, Spotts S, Rajah G, Tesfay L, Schulz VS and **Miranti CK** (2009) Mechanism of Metastasis Suppression by KAI1/CD82: Integrins, Tetraspanins, and Suppression of c-Met Activation. **Beatson International Cancer Conference: Microenvironment, Motility, and Metastasis**, Glasgow, Scotland, July 5-8.

MECHANISM OF METASTASIS SUPPRESSION BY KAI1/CD82: INTEGRINS, TETRASPANINS, AND SUPPRESSION OF C-MET ACTIVATION

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The mechanism by which the metastasis suppressor gene, KAI1/CD82, suppresses metastasis remains unsolved. KAI1/CD82 is a member of the tetraspanin family, whose members work together to regulate integrins. Previous studies in our lab demonstrated that KAI1/CD82 suppresses the activity of the receptor tyrosine kinase c-Met, which is known to promote migration and invasion of tumor cells. Furthermore, expression of KAI1/CD82 is sufficient to completely suppress c-Met -dependent metastasis and activation in HGF-transgenic mice. The extent to which suppression of c-Met by KAI1/CD82 is regulated by tetraspanin-dependent interactions with integrins was investigated. The tetraspanin CD151 is known to bind directly to $\alpha3\beta1$ integrin and to promote integrin-dependent cell migration. CD151 also associates with other tetraspanins such as KAI1/CD82 and CD9. Thus, re-expression of CD82 in CD151-expressing migratory cells might negatively impact CD151. Therefore, our hypothesis was that interactions between CD151/$\alpha3\beta1$ and CD82 are required to suppress c-Met activity. We found that siRNA-mediated loss of tetraspanin CD9 and CD151 blocked the ability of CD82 to suppress c-Met activation, but not invasion. Loss of $\alpha3$ or $\alpha6$ integrin had no effect on the ability of CD82 to suppress c-Met. Loss of CD151, $\alpha3$, or $\alpha6$ cooperated with loss of CD82 to suppress invasion. In addition, the presence of CD82 in cells decreased the levels of CD9 and CD151 association with $\alpha3\beta1$, and promoted the association of CD151/$\alpha6\beta1$/c-Met. Thus, we propose that CD82-mediated suppression of c-Met requires CD151, which sequesters $\alpha6\beta1$ and c-Met, and dissociates $\alpha3\beta1$ from other tetraspanins and c-Met, to limit c-Met and integrin activation.

Park E and **Miranti CK** (2010) Regulation of E-Cadherin and Cell-Cell Adhesion by the Metastasis Suppressor Tetraspanin KAI1/CD82. **Metastasis and the Tumor Microenvironment (AACR/MRS Joint Meeting)**, Philadelphia, PA, Sept 12-15.

Regulation of E-Cadherin and cell-cell adhesion by the metastasis suppressor Tetraspanin KAI1/CD82

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One of the characteristic features of metastatic disease is the loss of normal cell-cell adhesion. Classical cadherins, including E-cadherin, are a family of transmembrane proteins that are critical for cell-cell adhesion, and regulate the three dimensional structure of tissues, including polarized epithelia by establishing adherens junctions. In cancers of epithelial origin, loss of E-cadherin mediated cell-cell contact due to alterations in cadherin function or expression effectively frees cells of their neighbors, disrupting normal epithelial organization and potentially allowing migration out of the primary tumor.

The tetraspanin protein KAI1/CD82 was first characterized as a metastasis suppressor in prostate cancer; loss of CD82 is observed in many different human cancers, and its loss correlates with poor prognosis. Re-expression of CD82 in metastatic tumor cells lines has been demonstrated to decrease migration and invasion of tumor cells, in addition to increasing cell-cell adhesion. The mechanism by which CD82 enhances cell-cell adhesion is unknown. CD82 and other tetraspanins and tetraspanin-associated proteins form microdomain complexes within the plasma membrane, where they are thought to control the organization and function of different signaling complexes. Our hypothesis is that loss of CD82 promotes the reduction in E-cadherin based cell-cell adhesion in metastatic tumor cells, either by down-regulating E-cadherin cell surface expression or through disruption of adherens junction by altering the protein composition of the tetraspanin complex that associates with E-cadherin.

We have stably re-expressed CD82 in the metastatic prostate cancer cell line DU145; tumor cells isolated from a brain metastasis. CD82 expression was found to dramatically enhance E-cadherin-dependent cell adhesion in DU145 cells. Furthermore, in immortalized prostate epithelial cells expressing endogenous CD82 or in DU145 cells expressing ectopic CD82, CD82 and E-cadherin localize to similar structures in cells forming cell-cell contacts, but not at stable cell-cell junctions. Using density gradient fractionation and immunoprecipitation of CD82 from these cells, we have isolated a complex from tetraspanin-enriched microdomains comprised of CD82, CD9, E-cadherin, EWI proteins, and β-catenin. We hypothesize that this complex is required for the formation of E-cadherin mediated cell-cell junctions, and that restoration of CD82 will decrease motility, invasion, and metastasis by restoring normal cell-cell adhesion.

Saari KM, Spotts SM, Park ER, Rajah G, Schulz VS and **Miranti CK** (2011) CD82/KAI1
Suppresses Prostate Cancer Metastasis via Inhibition of the Receptor Tyrosine Kinase
Met**. Innovative Minds in Prostate Cancer Today – IMPaCT Meeting**. Orlando, FL,
Mar 9-12.

**CD82/KAI1 Suppresses Prostate Cancer Metastasis via Inhibition of the Receptor
Tyrosine Kinase Met**

Kristen M. Saari, Susan M. Spotts, Electa R. Park, Gary Rajah, Veronique S. Schulz, and Cindy
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Background: Expression of the tetraspanin protein, CD82/KAI1, is lost in metastatic tumors
from prostate cancer patients and its loss correlates with poor prognosis. The receptor tyrosine
kinase Met is over expressed in metastatic prostate cancers and promotes cell migration and
invasion. We demonstrated that re-expression of CD82 at physiological levels in metastatic
prostate tumor cell lines, suppresses integrin- or HGF-mediated activation of Met and that Met is
required for invasion. We further investigated the role of CD82 in suppressing Met activity *in
vivo* and determined the mechanism by which CD82 suppresses Met activity. We hypothesized
that CD82 alters the interaction between integrins and other tetraspanin molecules to suppress
Met activation.
Methods: Wild-type CD82 or an extracellular deletion mutant were re-expressed in PC3 and
DU145 metastatic prostate tumor cell lines, and the effects on HGF-induced Met activation,
association with integrins $\alpha3\beta1$ and $\alpha6\beta1$ and tetraspanins CD9 and CD151, Matrigel invasion,
and metastasis *in vivo* were assessed. The effect of siRNA-mediated loss of $\alpha3\beta1$, $\alpha6\beta1$, CD9,
or CD151 expression on Met activation and invasion was determined. Conditional loss of CD82
in the mouse prostate in combination with Pten loss was used to test the hypothesis that loss of
CD82 is sufficient to generate metastatic lesions from primary prostate tumors that develop in
these mice.
Results: CD82, CD9, and CD151 were found to associate with integrins $\alpha3\beta1$ and $\alpha6\beta1$.
Integrins $\alpha3\beta1$ and $\alpha6\beta1$ and CD151 were all required for efficient HGF-induced Met activation
and Matrigel invasion. Expression of CD82 caused a shift in tetraspanin/integrin complexes,
such that integrin $\alpha3$ was no longer associated with CD9 and CD151, and $\alpha6$ no longer
associated with CD9. Mutation of the extracellular domain of CD82 generated a molecule that
failed to inhibit Met activation. Orthotopic injection of DU145 cells into SCID mice over
expressing human-specific HGF results in 100% induction of prostate cancer metastases,
compared to no metastases after injection into normal SCID mice. CD82 re-expression in
DU145 cells suppresses prostate cancer metastasis by 95%, but has no effect on primary tumor
growth. Activated Met could be detected in primary and metastatic tumors from HGF-SCID
mice, while activated Met was not detected in primary tumors from CD82-expressing cells. We
are currently determining whether the CD82 deletion mutant is no longer capable of suppressing
metastasis *in vivo*. When CD82 conditional null mice were crossed to the prostate-specific Pten
conditional null mice, primary tumors were produced at the same frequency as in Pten mice, but
none of the mice developed metastasis. We are currently exploring the contribution of other
tumor metastasis suppressors in combination with CD82 to generate prostate cancer
metastases.
Conclusions: Together these data suggest that the extracellular domain of CD82 is required to
displace $\alpha3/\alpha6$ integrins from CD9/CD151 complexes, which is required to suppress Met
activation. While loss of CD82 may contribute to metastasis, it alone is not sufficient. Additional
molecular events are also required.
Impact: Understanding the molecular events associated with the development of metastatic
disease is required to identify potential biomarkers and effective therapeutic strategies.

Saari KM, Edick MJ, Sian KR and Miranti CK (2009) Development of a Total Kai1/CD82 Murine Knockout. **9th Annual Symposium Michigan Prostate Research Colloquium**, Wayne State University, Detroit, MI, May 30.

DEVELOPMENT OF A TOTAL KAI1/CD82 MURINE KNOCKOUT

Kristen M. Saari[1], Mathew J. Edick[1], Katie R. Sian[2], and Cindy K. Miranti[1]
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KAI1/CD82, a nearly ubiquitous transmembrane tetraspanin protein in mammals, was first identified as a metastasis suppressor in a rat prostate cancer model and has since been shown to play the same role in many cancers and cell lines. Our study, which investigates the effects of CD82 elimination in normal cells, is the counterpart to most experiments, which reintroduce CD82 into invasive cancer cells where its expression has been lost. The objective of our project is to develop a total murine knockout, in which expression of CD82 is deleted in all tissues. Using the Cre/LoxP system, we targeted exons IV and V to be deleted and included a neo cassette for positive selection and a viral thymidine kinase cassette for negative selection of homologous recombinants. Our construct was transfected into embryonic stem cells by electroporation with the surviving clones being screened for the correct homologous recombination by PCR, sequencing, and Southern Blotting. After two electroporations, two out of five positive homologous recombinants were injected into mice blastocysts, which in turn produced four male chimeric offspring. Two chimeras backcrossed to C57/BL6 wild type females went germline and produced 100% agouti mice. Male homozygous CD82 floxed mice crossed with female CMV-Cre mice produced a conventional CD82 total knockout mouse. We have successfully deleted Exon IV and V (DNA and mRNA), and cannot detect functional CD82 protein in the liver, kidney or prostate. There was not a compensatory increase in tetraspanins CD9, CD81 or CD151 in these tissues. The CD82 null mice are not embryonic lethal, show no breeding phenotype, nor have any other visual phenotypic defects. We are currently investigating the total CD82 knockout mice for any blood or blood vessel defects via a tail bleeding assay, Matrigel plug angiogenesis and oxygen-induced retinopathy. We are also interested in looking at CD82 knockout in the context of Pten or APC prostate-specific knockout for a potential metastasis model.

Saari KM, Edick MJ, Sian KR and Miranti CK (2010) Development and Investigation of a Total KA11/CD82 Murine Knockout. **10th Annual Symposium Michigan Urological Oncology Research Colloquium**, University of Michigan, Ann Arbor, MI, June 11-12.

DEVELOPMENT AND INVESTIGATION OF A TOTAL KAI1/CD82 MURINE KNOCKOUT
Saari, KM

To gain a better understanding of the function of CD82 in mammalian cells, we generated a conditional genetic deletion in the mouse genome. KAI1/CD82, a nearly ubiquitous transmembrane tetraspanin protein in mammals, was first identified as a metastasis suppressor in a rat prostate cancer model and has since been shown to play the same role in many cancers and cell lines. Our study, which investigates the effects of CD82 elimination in normal cells, is the counterpart to most experiments, which reintroduce CD82 into invasive cancer cells where its expression has been lost. The objective of this project is to develop and investigate a total murine knockout, in which expression of CD82 is conditionally deleted in all tissues using the Cre/LoxP system. Our construct was transfected into 129/SvJ embryonic stem cells, with the surviving clones being screened for the correct homologous recombination by PCR, sequencing, and Southern Blotting. After electroporation, the positive clones were injected into C57/BL6 blastocysts producing four male chimeric offspring, two of which went germline upon backcrossing to C57/BL6 wild type (WT) females. Male homozygous CD82 floxed mice crossed with female CMV-Cre mice produced conventional CD82 total knockout (KO) mice. We have successfully deleted Exon IV and V (DNA and mRNA), and cannot detect functional CD82 protein in the liver, kidney or prostate. There was not a compensatory increase in tetraspanins CD9, CD81 or CD151 in these tissues. The CD82 KO mice are viable, healthy and fertile with normal tissue morphology. Further investigation of angiogenesis, using an oxygen-induced retinopathy model, revealed normal retinal vasculature in the CD82 KO mice, while a Matrigel plug assay showed a 22% greater Matrigel weight in the KO mice compared to WT controls. However, we could not conclude this was due to an increased vasculature via CD34 staining of neovasculature in the Matrigel plugs. Tail bleeding revealed that CD82 KO mice clot significantly faster than WT controls, so we are currently searching for specific platelet defects that could explain this. Lastly, we are investigating a potential metabolic defect by looking at kidneys via Transmission Electron Microscopy, as well as urine and fecal water content. We are also exploring if there are any strain-dependent effects by investigating CD82 KO mice in a pure FVB/N or c57BL6 background. Lastly, we are pursuing a prostate metastasis model by creating CD82 KO mice in the context of Pten or APC prostate-specific KO mice.

Park E and Miranti CK (2009) Tetraspanin Kai1/CD82 Regulation of Cell-Cell adhesion: A Mechanism of Metastasis Suppression? **9th Annual Symposium Michigan Prostate Research Colloquium**, Wayne State University, Detroit, MI, May 30.

TETRASPANIN KAI1/CD82 REGULATION OF CELL-CELL ADHESION: A MECHANISM OF METASTASIS SUPPRESSION?

Electa Park and Cindy Miranti,
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One of the molecular features of metastatic disease is the loss of cell-cell adhesion. Adhesion of cells to themselves, as well as to the tissue, prevents their dissociation and maintains tissue integrity. However, during metastasis, cells lose this tissue association and become free to move about the body where they can set up secondary tumors at distant sites. Classical cadherins, including E-cadherin (Epithelial cadherin), N-cadherin (Neuronal cadherin), and P-cadherin (Placental cadherin) are a family of transmembrane proteins that are critical for cell-cell adhesion, and regulate the three dimensional structure of tissues, including polarized epithelia. In cancers of epithelial origin, loss of E-cadherin mediated cell-cell contact due to alterations in cadherin function or expression effectively frees cells of their neighbors, potentially allowing migration out of the primary tumor.

The tetraspanin protein KAI1/CD82 was first characterized as a metastasis suppressor in prostate cancer; loss of CD82 is observed in many different human cancers, including prostate, breast, colon, lung, ovarian, and pancreatic malignancies, where it correlates with poor prognosis. Re-expression of CD82 in metastatic tumor cells lines has been demonstrated to increase cell-cell adhesion of tumor cells. The mechanism by which CD82 enhances cell adhesion is unknown. Thus, one possibility is that loss of CD82 promotes the reduction in E-cadherin based cell-cell adhesion in metastatic tumor cells, which may explain how loss of CD82 promotes cancer metastasis.

The prostate cancer cell lines PC-3 and DU145 were isolated from bone and brain metastases respectively, and model aggressive, invasive prostate cancer. While PC-3 cells have lost expression of E-cadherin and its complex partner a-catenin, they do express N-cadherin, and the non-classical, osteoblast-specific cadherin-11. DU145 cells retain some expression of E-cadherin, as well as its downstream binding partner's a- and b-catenin. We hypothesize that CD82 re-expression in metastatic prostate cancer cell lines will lead to increased N-and E-cadherin mediated cell-cell adhesion, resulting in decreased motility and invasion in vitro and decreased metastasis in vivo. To investigate this hypothesis, we will generate PC-3 and DU145 cell lines that stably express wild-type and mutant CD82 and examine the effect on cell-cell adhesion, adherens junction and actin organization, in vitro migration and invasion, and in vivo metastasis.

Park E and Miranti CK (2009) Tetraspanin KAI1/CD82 Regulation of Cell-Cell Adhesion: A Mechanism of Metastasis Suppression? **Gordon Research Conference: Cell Contact and Adhesion**, Waterville Valley, NH, June 28-July 3.

TETRASPANIN KAI1/CD82 REGULATION OF CELL-CELL ADHESION: A MECHANISM OF METASTASIS SUPPRESSION?

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One of the molecular features of metastatic disease is the loss of cell-cell adhesion. Adhesion of cells to themselves, as well as to the tissue, prevents their dissociation and maintains tissue integrity. However, during metastasis, cells lose this tissue association and become free to move about the body where they can set up secondary tumors at distant sites. Classical cadherins, including E-cadherin (Epithelial cadherin), N-cadherin (Neuronal cadherin), and P-cadherin (Placental cadherin) are a family of transmembrane proteins that are critical for cell-cell adhesion. In cancers of epithelial origin, loss of E-cadherin mediated cell-cell contact due to alterations in cadherin function or expression effectively frees cells of their neighbors, potentially allowing migration out of the primary tumor. The tetraspanin protein KAI1/CD82 was first characterized as a metastasis suppressor in prostate cancer; loss of CD82 is observed in many different human cancers, including prostate, breast, colon, lung, ovarian, and pancreatic malignancies, where it correlates with poor prognosis. Re-expression of CD82 in metastatic tumor cells lines has been demonstrated to increase cell-cell adhesion of tumor cells. The mechanism by which CD82 enhances cell adhesion is unknown. Thus, one possibility is that loss of CD82 promotes the reduction in E-cadherin based cell-cell adhesion in metastatic tumor cells, which may explain how loss of CD82 promotes cancer metastasis. The prostate cancer cell lines PC-3 and DU145 were isolated from bone and brain metastases respectively, and model aggressive, invasive prostate cancer. While PC-3 cells have lost expression of E-cadherin and its complex partner α-catenin, they do express N-cadherin, and the non-classical, osteoblast-specific cadherin-11. DU145 cells retain some expression of E-cadherin, as well as its downstream binding partners, α- and β-catenin. We hypothesize that CD82 re-expression in metastatic prostate cancer cell lines will lead to increased N-and E-cadherin mediated cell-cell adhesion, resulting in decreased motility and invasion *in vitro* and decreased metastasis *in vivo*. To investigate this hypothesis, we will generate PC-3 and DU145 cell lines that stably express wild-type and mutant CD82 and examine the effect on cell-cell adhesion, adherens junction and actin organization, *in vitro* migration and invasion, and *in vivo* metastasis.

Park E and Miranti CK (2010) Regulation of E-cadherin and Cell-Cell Adhesion by the Tetraspanin CD82. **10th Annual Symposium Michigan Urological Oncology Research Colloquium**, University of Michigan, Ann Arbor, MI, June 11-12.

Regulation of E-cadherin and Cell-Cell Adhesion by the Tetraspanin CD82

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One of the characteristic features of metastatic disease is the loss of normal cell-cell adhesion. Classical cadherins, including E-cadherin (Epithelial cadherin), are a family of transmembrane proteins that are critical for cell-cell adhesion, and regulate the three dimensional structure of tissues, including polarized epithelia by establishing adherens junctions. In cancers of epithelial origin, loss of E-cadherin mediated cell-cell contact due to alterations in cadherin function or expression effectively frees cells of their neighbors, disrupting normal epithelial organization and potentially allowing migration out of the primary tumor.

The tetraspanin protein KAI1/CD82 was first characterized as a metastasis suppressor in prostate cancer; loss of CD82 is observed in many different human cancers, and its loss correlates with poor prognosis. Re-expression of CD82 in metastatic tumor cells lines has been demonstrated to decrease migration and invasion of tumor cells, in addition to increasing cell-cell adhesion. The mechanism by which CD82 enhances cell-cell adhesion is unknown; one hypothesis is that loss of CD82 promotes the reduction in E-cadherin based cell-cell adhesion in metastatic tumor cells, either by down-regulation of E-cadherin cell surface expression or disruption of adherens junction through alterations in the protein composition of the tetraspanin complex that associates with CD82.

We have stably re-expressed CD82 in the metastatic prostate cancer cell line DU145; tumor cells isolated from a brain metastasis that retains expression of E-cadherin and its binding partners α- and β-catenin. In immortalized prostate epithelial cells expressing endogenous CD82 or in DU145 cells expressing ectopic CD82, CD82 and E-cadherin localize to similar structures in cells forming or breaking cell-cell contacts, but not at stable cell-cell junctions. Using density gradient fractionation and immunoprecipitation of CD82 from these cells, we have isolated a complex from tetraspanin-enriched microdomains comprised of CD82, CD9, E-cadherin, EWI proteins, and β-catenin. We hypothesize that this complex is required for the formation and/or dissolution of E-cadherin mediated cell-cell junctions, and that restoration of CD82 will result in increased E-cadherin mediated cell-cell adhesion, and consequently decreased motility and invasion *in vitro*.

Park E and Miranti CK (2010) CD82 Regulation of E-Cadherin: A Mechanism of Metastasis Suppression. **Society for Basic Urological Research Annual Meeting**, Atlanta, GA, Nov 11-14.

CD82 REGULATION OF E-CADHERIN: A MECHANISM OF METASTASIS SUPRESSION
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One of the characteristic features of metastatic disease is the loss of normal cell-cell adhesion. Classical cadherins, including E-cadherin, are a family of transmembrane proteins that are critical for cell-cell adhesion, and regulate the three dimensional structure of tissues. In cancers of epithelial origin, loss of E-cadherin mediated cell-cell contact effectively frees cells of their neighbors, disrupting normal epithelial organization and potentially allowing migration out of the primary tumor.

The tetraspanin protein KAI1/CD82 was first characterized as a metastasis suppressor in prostate cancer; loss of CD82 is observed in many different human cancers, and its loss correlates with poor prognosis. Re-expression of CD82 in metastatic tumor cells lines has been demonstrated to decrease migration and invasion of tumor cells, in addition to increasing cell-cell adhesion. The mechanism by which CD82 enhances cell adhesion is unknown; one hypothesis is that loss of CD82 promotes the reduction in E-cadherin based cell-cell adhesion in metastatic tumor cells, either by regulation of E-cadherin cell surface expression or disruption of adherens junction through alterations in the protein composition of the tetraspanin web. Alternatively, EGFR signaling induces internalization and recycling of E-cadherin, and CD82 suppresses EGFR activation. Loss of CD82 may potentiate EGFR signaling, and hence E-cadherin internalization, providing an indirect mechanism for CD82 regulation of cell-cell adhesion.

We stably re-expressed CD82 in the metastatic prostate cancer cell line DU145. In these cells, CD82 and E-cadherin localize to sites of forming or breaking cell-cell contacts, but not stable cell-cell junctions. Using density gradient fractionation and immunoprecipitation of CD82 from these cells, we have isolated a complex from tetraspanin-enriched microdomains comprised of CD82 and E-cadherin. Additionally, preliminary data suggest that mutant CD82 lacking the second loop of the large extracellular domain is unable to suppress EGFR signaling in adherent cells. We hypothesize that a CD82/E-cadherin complex is required for the formation and/or dissolution of E-cadherin junctions, and that restoration of CD82 will result in increased E-cadherin based cell-cell adhesion, via a direct or indirect mechanism.

Park E and Miranti CK (2011) CD82 Regulation of E-cadherin: A Mechanism of Metastasis Suppression. **FASEB: Membrane Organization by Molecular Scaffolds**, Saxtons River, VT, July 17-22.

CD82 REGULATION OF E-CADHERIN: A MECHANISM OF METASTASIS SUPRESSION

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The tetraspanin protein KAI1/CD82 is a non-enzymatic molecular scaffold that was first characterized as a metastasis suppressor in prostate cancer; loss of CD82 is observed in many different human cancers, and its loss correlates with poor prognosis. E-cadherin is a calcium-dependent cell-cell adhesion molecule that regulates epithelial cell polarity, and is the main component of the cell-cell adhesion structures known as adherens junctions. Loss of both normal cell-cell adhesion and epithelial apical/basal polarity are required for metastatic progression of cancer. For prostate cancer, as with many other epithelial malignancies, disease *in situ* is highly treatable and recurrence rates are low; however metastatic disease is frequently difficult to treat and often lethal. Re-expression of CD82 in prostate cancer cells lines has been demonstrated to decrease the metastatic behaviors of migration and invasion in tumor cells, in addition to increasing cell-cell adhesion. The mechanism by which CD82 enhances cell-cell adhesion is unknown; one hypothesis is that loss of CD82 promotes the reduction in E-cadherin based cell-cell adhesion in metastatic tumor cells, either by a direct scaffolding interaction regulating E-cadherin cell surface expression, or by disruption of adherens junction organization through alterations in the protein composition of the tetraspanin web. Alternatively, E-cadherin internalization and recycling is regulated by EGFR signaling, and CD82 suppresses EGFR activation by inhibiting receptor dimerization. Loss of CD82 may potentiate EGFR signaling, and hence E-cadherin turnover and internalization, providing an indirect mechanism for CD82 regulation of cell-cell adhesion.

We stably re-expressed CD82 in the metastatic prostate cancer cell line DU145. In these cells, CD82 and E-cadherin localize to sites of forming or breaking cell-cell contacts, but not stable cell-cell junctions. Using density gradient fractionation and immunoprecipitation of CD82 from these cells, we have isolated a complex from tetraspanin-enriched microdomains comprised of CD82 and E-cadherin. Additionally, preliminary results indicate that CD82 re-expression may increase E-cadherin functionality, based on adhesion of cells to a recombinant E-cadherin:Fc matrix. A mutant of CD82 lacking the second loop of the large extracellular domain may potentiate E-cadherin function. We hypothesize that a CD82/E-cadherin complex is required for proper molecular organization during the formation and/or dissolution of adherens junctions, and that restoration of CD82 will result in increased E-cadherin based cell-cell adhesion and decreased metastatic behaviors, via either a direct or indirect mechanism.

Additionally, the mechanism by which CD82 expression is regulated in normal and cancer cells remains largely unknown. CD82 can be ubiquitinated by the E3 ligase gp78 (Tsai et al 2007), a 7-transmembrane glycoprotein with a cytosolic E3 ligase domain; ubiquitination leads to proteasomal degradation of CD82. Intriguingly, gp78 is up regulated in metastatic cancers, and gp78 and CD82 seem to have an antagonistic relationship, as high levels of one correlate with low levels of the other. We hypothesize that CD82 is ubiquitinated on its cytosolic face, and that mutation of the cytosolic lysines, the putative ubiquitination targets, will stabilize CD82 expression and enhance interactions with tetraspanin-enriched microdomains and E-cadherin. We have generated CD82 mutant constructs (CD82 KR mutants) in which the cytosolic lysines, either alone or in combination, have been mutated to arginine. Using stable pools of DU145 cells, we have analyzed the expression of the CD82 KR mutants as well as their inclusion in tetraspanin-enriched microdomains. Our preliminary results suggest that mutation of lysines 7, 10, and 266 may stabilize CD82, while mutation of lysine 263 may destabilize

CD82 expression. Furthermore, we find that mutation of lysines 7 and 10 may increase association with E-cadherin. We are currently mapping the specific ubiquitination sites on CD82, as well as analyzing the functional consequences of these mutations on CD82 interactions.

PUBLICATION

Miranti, C.K. 2009. Controlling cell surface dynamics and signaling: how CD82/KAI1 suppresses metastasis. **Cellular Signaling** 21:196–211.
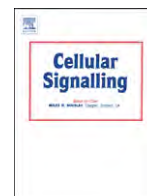
Review

Controlling cell surface dynamics and signaling: How CD82/KAI1 suppresses metastasis

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ARTICLE INFO

ABSTRACT

The recent identification of metastasis suppressor genes, uniquely responsible for negatively controlling cancer metastasis, are providing inroads into the molecular machinery involved in metastasis. While the normal function of a few of these genes is known; the molecular events associated with their loss that promotes tumor metastasis is largely not understood. KAI1/CD82, whose loss is associated with a wide variety of metastatic cancers, belongs to the tetraspanin family. Despite intense scrutiny, many aspects of how CD82 specifically functions as a metastasis suppressor and its role in normal biology remain to be determined. This review will focus on the molecular events associated with CD82 loss, the potential impact on signaling pathways that regulate cellular processes associated with metastasis, and its relationship with other metastasis suppressor genes.

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1. Introduction

Metastatic cancer remains an incurable disease. The successful identification and analysis of tumor suppressor genes responsible for the initiation of primary tumors, and identification of the genes that they suppress, has been key to the successful development of new cancer therapies. It is logical to propose that a similar approach, i.e. identification of metastasis suppressor genes, would be equally beneficial to curing metastatic disease. Indeed, such genes exist; over 20 metastasis suppressor genes have been identified based on their specific ability to suppress metastasis, but not primary tumor growth, in xenograft models [1,2]. Many follow-up studies in human cancer tissues support their role as metastasis suppressors, as loss of expression is observed almost exclusively in metastatic disease. The normal biological function of several, but not all, metastasis genes are known; however, few have been characterized with respect to how their loss promotes tumor metastasis. It is not even known if loss of any of these genes in vivo is sufficient or absolutely required for metastasis. This review will focus on what is known about the metastasis suppressor gene, KAI1/CD82. Despite intense scrutiny, many aspects of how CD82 specifically functions as a metastasis suppressor and its role in normal biology remain to be determined.

1.1. Establishment of KAI1 (CD82) as a metastasis suppressor

The metastasis suppressor function of KAI1/CD82 was first detected in a genetic screen using the metastatic rat AT6.1 prostate cancer cell line expressing fragments of chromosome 11, on which CD82 is located [3]. Re-expression of CD82 in AT6.1 cells and subsequent subcutaneous injection into nude mice significantly reduced metastases without affecting primary tumor growth. Numerous xenograft studies using other metastatic cell lines, including MDA-MB-435, breast LCC6, liver MHCC97-H, lung LLC, HT1080 sarcoma, and prostate LNCaP, further confirmed the metastasis suppressor function of CD82 [4–9]. The route of injection, subcutaneous, orthotopic, or tail vein, did not impact the outcome and no major effects on primary tumor growth were seen in any model.

That CD82 is a valid metastasis suppressor gene is further supported by numerous clinical studies. Loss of CD82, both protein and mRNA, is strongly correlated with poor prognosis in many malignancies (reviewed in [10]), including prostate, colon, lung, pancreatic, breast, ovarian, and several others. While the direct association between CD82 and metastasis per se is not always straight forward, the number of reports citing CD82 loss out-numbers five to one those reporting an increase or no change. In two prostate cancer mouse models, down regulation of CD82 expression was observed in metastatic disease (CKM unpublished data)[11]. Thus CD82 loss is highly correlative with metastatic disease, CD82 loss occurs at the transcriptional level, and based on xenograft animal models CD82 suppresses metastasis. Furthermore, CD82 loss is likely to be a universal event in the development of metastasis since it is observed in many types of cancer.

1.2. CD82 molecular biology

CD82 was first cloned based on its affinity for several monoclonal antibodies (R2, IA4, C33, 4F9) that recognize lymphocytic surface antigens [12–16]. CD82 was subsequently assigned membership in the Cluster of Differentiation antigens [17]. CD82 is a member of the 4-span transmembrane super family (TM4SF) of type III membrane proteins, specifically of the tetraspanin subgroup (Tspan). There are 33 tetraspanins in the human genome (Table 1). Tetraspanin proteins are not present in yeast or bacteria, but are present in fungi and all multicellular organisms [18]. Direct functional comparisons between divergent species are difficult because of low conserved DNA sequence homology.

CD82 and several other tetraspanins are fairly ubiquitously expressed. Northern analysis of human tissues reveals high expression of CD82 in the spleen, thymus, prostate, ovary, small intestine, colon,

Table 1
Tetraspanin family genes, tissues expressed, and identified functions

Tspan	Alt. names	Tissues	Function
1	NET-1	Liver cancer	Tumorigenesis
2	?	CNS	Oligodendrocyte differentiation
3	OAP-1, TM4SF8	Oligodendrocytes	Migration, proliferation
4	NAG-2, TM4SF7	Fibroblasts	Unkn
5	NET-4, TM4SF9	CNS	Unkn
		Osteoclasts	Osteoclastogenesis
6	TM4SF6	?	X-linked gene
7	TM4SF2, A15	Neurons	Mental retardation
8	CO-029, D6.1A	Colon cancer	Metastasis, angiogenesis
9	NET-5	Ovarian cancer	Amplified 12p gene
10	OCSP, oculospanin	Pigment epithelium	?
11	?	?	?
12	NET-2	?	?
13	NET-6	Breast cancer	Tumor suppressor
		Osteoclasts	Osteoclastogenesis
14	?, TM4SF14	?	?
15	NET-7	?	?
16	TM-8, TM4-B	?	?
17	?, TM4SF17	?	?
18	Neurospanin	Brain, CNS	?
19	?	?	?
20	Uroplakin1b	Bladder	Urine permeability barrier
21	Uroplakin1a	Bladder	Urine permeability barrier
22	RDS/peripherin	Rods/cones	Retinal structure
23	ROM1	Rods	Survival of photoreceptors
24	CD151	Endothelial cells	Angiogenesis
		Platelets	Thrombi stabilization
		Kidney	Organ structure
		Epithelial	Wound healing
25	CD53/OX44	Immune cells	B-cell signaling
		Neutrophils	Immune surveillance
26	CD37	Immune cells	T/B-cell signaling
27	CD82	Epithelial cells	Tumor metastasis suppressor
		Immune cells	T-cell signaling
28	CD81	Oocytes	Egg/sperm fusion
		Immune cells	B-cell signaling
		Brain	Astrocyte/glia proliferation
		Pigment pithelia	Proliferation
29	CD9	Oocytes	Egg/sperm fusion
		Immune cells	T/B-cell function
		PNS	Myelination
30	CD63	Ubiquitous	Protein trafficking
31	SAS	Sarcoma cells	Sarcoma amplified sequence
32	TSSC6	Immune cells	T-cell proliferation
		Platelets	Thrombi stabilization
33	Penumbra	Erythroblasts	Erythropoiesis

placenta, lung, liver, kidney, and pancreas. Significantly, lower expression is seen in the heart, brain, muscle, and testis [3]. Mouse CD82 mRNA was highest in the spleen, kidney, lung, and liver [19], with a similar distribution seen in the rat [20]. Immunostaining of mouse tissues reveals distinct subtissue distributions. Mouse CD82 protein is primarily localized to spleen lymphoid tissue, medullary collecting ducts and distal convoluted tubules of the kidney, arteriolar smooth muscle of the lung, hepatocytes and sinusoidal lining of the liver, islet cells, and is found in many epithelial cells including epididymus, prostate; colon; bladder, ureter, urethra, uterus, ovary, oviducts, testes, and seminal vesicles. CD82 was also present in most vascular endothelium except arterioles and the brain [21].

1.2.1. Transcriptional regulation

There is little evidence for gene mutation, loss of heterozygosity, promoter mutation, or hypermethylation to explain the loss of CD82 expression in clinical isolates of metastatic cancers [22–28]. Altered transcription or splice variants remain as possible mechanisms for loss of CD82 mRNA. One splice variant in which exon 7 is deleted has been reported [29]. Spliced KAI1 mRNA was detected in metastatic and invasive tissues of gastric and bladder cancers as well as several cell lines [29,30]. However, the level of spliced transcript was present at several-fold lower levels than full length mRNA, and did not correlate well with invasiveness or metastasis. Thus its significance in the etiology of metastasis remains unknown.

The human CD82 promoter is G-C rich and Tata-less, and contains an array of potential promoter elements including Sp1, AP-2, GATA-1, PEA3, NF-IL6, MEP1, Myb, TCF-1, HNF3, NF-1, zeste, and Ets binding sites [31,32]. The mouse promoter contains many of the same putative promoter elements [19]. Several extracellular stimuli have been reported to enhance CD82 expression and include cytokines (IL-1β, IL-4, IL-6, IL-13, IFN-γ, TNF-α), growth factors (NGF), phorbol esters (PMA), drugs (Genistein, etoposide), and 8-bromo-cAMP [11,33–39]. The mechanisms by which these stimuli regulate CD82 transcription are virtually unknown, except that NF-κB is known to mediate some of the effects of the cytokines.

Promoter deletion analysis initially identified three transcriptional regions in the CD82 promoter; an enhancer region (−922 to −846); a negative regulatory region (−735 to −197); and the minimal promoter (−197 to +351) [10,32,40]. A p53-like regulatory element, responsible for etoposide induction of CD82, is located at −860 in the enhancer region and was initially intriguing given that loss of p53 in prostate cancer is a late event correlating with progression to metastasis. Despite the fact that over expressed p53 can bind to the promoter and enhance CD82 expression in transfected cells, the correlation between p53 loss and CD82 loss in clinical samples does not stand up, arguing against a strict one-to-one relationship [36,41,42]. Other reports suggest that various combinations of p53, AP2, or JunB binding at the extended AP2-p53-AP1 element are responsible for regulating full CD82 expression and binding of these factors may be differentially altered during metastasis [40,43].

Cytokine induced CD82 expression in immune cells is mediated primarily via NF-κB [44]. In two p53 mutant epithelial cell lines, TNFα-induced CD82 expression was also dependent on NF-κB [45]. Subsequently, ChIP analysis revealed that NF-κB p50, but not p65, is present on the CD82 promoter. The CD82 promoter recruits NF-κB p50, Bcl3 (functionally related to IκBα) and the N-CoR/TAB2/HDAC3 corepressor complex, which results in transcriptional inactivation. IL-1β stimulation transiently recruits Tip60 to p50 bound at the CD82 promoter, which is coincident with loss of the N-CoR complex, increased acetylation and phosphorylation of histones, and recruitment of Pol II. Over expression of a Tip60/Fe65/APP complex was sufficient to displace the N-Cor complex [46]. The Fe65 transcription activation domain binds to the nucleosome assembly factor SET, which is required for Fe65-mediated transactivation. ChIP experiments demonstrated that a complex including Fe65/APP/Tip60 and

SET is associated with the CD82 promoter. SET is required for full levels of CD82 transcription.

However, the Tip60 coactivator complex was not recruited to the CD82 promoter in metastatic prostate cancer cells due to low levels of Tip60 expression in these cells [8]. IL-1β-induced Tip60 expression and recruitment could be restored by inhibiting β-catenin expression. A reptin/β-catenin complex was detected at the CD82 promoter that was present only in metastatic cells. The reptin/β-catenin complex could be displaced after IL-1β stimulation by over expressing Tip60. Modulation of Tip60 or β-catenin levels in metastatic cells or normal cells respectively alters IL-β1-mediated matrigel invasion. It was proposed that high levels of the β-catenin–reptin complex, due to Wnt activation, and simultaneous down regulation of Tip60 act together to inhibit CD82 expression and drive metastasis. Subsequently it was shown that the reptin repressive function requires its sumoylation at Lys456 by SENP1/SUSP1 [47]. Blocking reptin sumoylation in metastatic prostate cells restored CD82 mRNA and decreased matrigel invasion.

A recent study in metastatic breast cancer cells suggests CD82 transcription can also be regulated at the level of genomic organization. SATB1 regulates gene expression by recruiting chromatin remodeling enzymes and transcription factors, and tethers multiple genomic loci via specialized DNA sequences to globally control transcription. RNAi-mediated knockdown of SATB1 in metastatic MDA-MB-231 breast cancer cells restored acinar polarity and inhibited tumor growth and metastasis in vivo. Intriguingly, CD82, in addition to several other known metastasis suppressor genes, including nm23, KiSS1, BRMS1, claudin 1, and E-cadherin, were coordinately up-regulated in the SATB1 deleted tumor cells [48]. Thus, loss of p53, enhanced Wnt/β-catenin signaling, stress activated Jnk, and increased expression of SATB1 could all work together or in various combinations to promote the loss of CD82 expression and metastasis.

1.2.2. Post-transcriptional regulation

The incomplete correlation between CD82 mRNA or protein loss and metastasis in some clinical specimens may reflect additional mechanisms for removing CD82 function. Inhibition of E3 ubiquitin ligase gp78 expression in highly metastatic HT1080 sarcoma cells suppressed metastasis, but had no effect on primary tumor growth [9]; an effect that mimics metastasis suppressor genes. CD82 was identified as a primary substrate of gp78. The E3 ligase activity of gp78 was required for its metastatic effects. Loss of gp78 resulted in increased CD82 expression and over expression of gp78 increased CD82 degradation. An inverse relationship between gp78 and CD82 expression was detected in human sarcoma tissue samples and inhibition of CD82 expression in gp78 negative cells restored metastasis. Thus, another possible mechanism for CD82 loss in metastatic tumors is its enhanced degradation or turnover.

Whether other post-transcriptional or translational mechanisms can account for loss of CD82 function in metastatic tumors remains to be addressed. At least two types of post-translational modifications, glycosylation and palmitoylation, are known to occur on CD82. Inhibition of these modifications affects CD82 function [49,50]. Whether the enzymes responsible for CD82 modification are altered in metastatic tumors is not known.

2. CD82 function

Tetraspanin proteins function in many aspects of cell physiology. Several excellent reviews provide extensive information on the genetics, structure, and function of tetraspanins [51–54]. Tetraspanins contain no intrinsic catalytic activity; therefore, current hypotheses favor a model whereby tetraspanins serve as master regulators of membrane organization, through interactions with surface molecules and each other. Through these interactions tetraspanins regulate a variety of cellular events including signaling, transcription, cell

adhesion, migration, survival, endo- and exocytosis, differentiation, and cell fusion. CD82 has been shown to be important for some these cellular processes, but specifically which ones are directly relevant to its metastasis suppressing ability has not been fully determined.

2.1. CD82 structure

The initial characterization of CD82 was aided by its sequence homology to several other tetraspanin proteins, namely CD81, CD9, CD63, and CD37 [12,14,15]. The assignment of CD82 to the tetraspanin family is based on the conserved structural motifs within this family. CD82 is a 267 amino acid type III membrane protein that spans the membrane 4 times, contains short N- and C-terminal cytoplasmic domains, a short 4 amino acid intracellular loop (IC), and two extracellular loops (EC1 and EC2). There is over 90% sequence conservation between human and mouse CD82 proteins within the 4 transmembrane domains, 81% in the cytoplasmic domains, but only 65% in EC2 [14,19]. Within the EC2 domain are three conserved sequence motifs, CCG, PXXC/PCXC, and a GC residue close to the 4th transmembrane domain. These motifs specifically distinguish tetraspanins from other TM4SF proteins [52,53]. The EC2 cysteine residues are predicted to form 2, 3, or 4 disulfide bonds, and are the basis for subclassification within the tetraspanin family [18,55].

Only the EC2 domain of CD81 has been crystallized [56]. Based on CD81, the EC2 structure of other tetraspanins including CD82, as well as the full length protein, were modeled [55,57–59]. The picture of CD82 that emerges is a short cytoplasmic 10 amino acid N-terminal amphipathic helix that lies parallel to the cell membrane and a relatively unstructured 14 amino acid C-terminal tail. The 4 trans-membrane domains are tightly packed left-handed antiparallel coiled coils. The 17 amino acid EC1 domain has a hydrophobic beta strand that nestles within a hydrophobic pocket of the EC2 domain. The EC2 domain has two alpha-helical bundles that extend directly up from the two transmembrane domains like a stalk, which supports a third helical domain as well as a highly variable region that is unique to each tetraspanin. CD82 EC2 domain is predicted to have three disulfide bonds within this variable region. The third helix lies parallel to the membrane causing the variable region to sit tilted relative to the stalk. The result is a tightly packed transmembrane protein whose prominent feature is an asymmetric EC2 domain protruding about 5 nm above the cell membrane. The cryo-EM structure of the bladder-specific tetraspanins, uroplakins, also support the notion of a rod like structure extending through the membrane with a prominent binding surface suspended above the membrane [60]. In the uroplakin complex, each tetraspanin protein is tightly associated with its single-pass transmembrane partner, and primarily associate with each other through interactions between the partner molecules, rather than the tetraspanins themselves.

2.2. CD82 in immune signaling

2.2.1. T-cells

The first defined function for CD82 was it role in regulating T-cell signaling through the T-cell receptor (TCR). TCR stimulation activates the src kinases, Fyn and Lck, following recognition of MHC-peptide complexes on antigen presenting cells. Lck and Fyn phosphorylate CD3, leading to recruitment and activation of ZAP-70. Subsequently, ZAP-70 phosphorylates adaptor molecules LAT, SLP-76, and Vav resulting in activation of PKC, MAPK, and Rho GTPases and ultimately transcription of genes such as IL-2 [61]. TCR signaling is maximal in the presence of costimulatory receptors. Monoclonal antibody binding to CD82 can serve as a costimulatory signal for full activation of T cells, and results in strong IL-2 and IFN-γ production and cell differentiation [62]. Costimulation of CD82 also induces cell adhesion, spreading, and actin polymerization [62,63]. Immobilization of CD82 with mono-clonal antibody was sufficient to induce tyrosine phosphorylation and

association of Vav1 with SLP76, which activates Rho GTPases. Global inactivation of Rho GTPases reduced CD82-induced cell spreading [64]. CD82 can be detected in a complex with TCR [65]. Both the cytoplasmic and extracellular regions of TCR-CD4 were required for CD82 interaction. CD82 initially colocalizes with TCR upon T-cell activation, but relocalizes with F-actin at the periphery of the immune synapse [66] and binding of Lck to TCR precludes CD82 binding [65]. Thus CD82, is involved in dynamic interactions during T-cell signaling.

The T-cell signaling data suggest that the primary function of CD82 in the costimulatory pathway is to facilitate events associated with actin polymerization, a function that is also attributed to integrins. Coincidently, CD82 associates with α4β1 integrin in T-cells [67]. CD82 levels on resting T-cells are low and during TCR activation there is a 3.5- to 7-fold increase in CD82 surface levels [37]. Increased CD82 expression is associated with enhanced homotypic interactions between T-cells [39]. Enhanced adhesion between T-cells was not due to CD82 directly, but was mediated by interactions between ICAM-1 and its integrin receptor LFA-1(αLβ2). CD82 and LFA-1 colocalized at cell–cell interaction points and could be coimmuno-precipitated from T-cells. LFA-1 antibodies could substitute for the CD82 costimulatory signal [68]. Thus the function of CD82 is to facilitate integrin-dependent events in T-cell signaling. Antibodies to other tetraspanins, CD9, CD53 or CD81, have similar costimulatory activity as CD82 antibodies; these tetraspanins also interact with integrins [69].

2.2.2. B-cells and antigen presentation

CD82 is also expressed on CD19+ B-cells [15]. Coimmunoprecipita-tion studies revealed that CD82 was present in complexes with MHCII receptors, B-cell costimulatory molecules CD19 and CD21, tetraspa-nins CD9, CD53, CD63, and CD81, and several integrins—α4β1, α6β1, and α5β1 [70–73]. Recent studies in CD81 null mice demonstrate that CD81 is essential for assembly and localization of the CD19 costimulatory complex in B-cells [74]. While CD9, CD53, and CD82 also form complexes with CD19/ CD21, their role in B-cell activation is still unknown. T-cell and B-cell activation is mediated by interactions with MHC molecules on antigen presenting cells (APCs). CD82 is also found in association with peptide-loaded HLA-DR MHC II complexes on intracellular membrane compartments in APCs. CD82 appears to facilitate transport and clustering of the loaded MHC II complexes on the cell surface [75].

2.2.3. Animal models

To date six tetraspanins have been deleted from the mouse genome. Each tetraspanin appears to have a unique biological role as well as overlapping roles with other tetraspanins (Table 1). For instance, only loss of CD9, and to a lesser extent loss of CD81, leads to a fertility defect due to impaired egg-sperm fusion [76–79]. CD81, but not CD9 or TSSC6, plays a critical role in B-cell activation by regulating expression of the BCR costimulatory molecule CD19 [80,81]. Both CD9 and CD81 are important in neurons, but in different cell types [82–84]. CD37, which is exclusively expressed in immune cells, is required for efficient signaling in both T- and B-cells [85,86] and penumbra (Tspan33) is required for efficient erythopoiesis [87]. Loss of either CD151 or TSSC6 leads to defects in platelets, while loss of CD151, but not TSSC6, also disrupts kidney function, impairs pathological angiogenesis, and inhibits wound healing [88–93]. Genetic deletion of CD82 has not yet been formally reported, but preliminary studies from our lab indicate that CD82 null mice are viable and fertile (Miranti, unpublished data). Phenotypes associated with loss of two or more tetraspanins await further analysis.

2.3. CD82 as a metastasis suppressor

Based on studies in immune cells, CD82 is important for cell signaling, cell adhesion, and sorting/trafficking of proteins to the cell

surface. Are these same functions relevant to its role in metastasis suppression, or are there additional functions for CD82 in non-immune cells? It is important to point out that although CD82 loss is associated with metastasis, up-regulation of other tetraspanins, namely CD151 and CO-029 is associated with more aggressive disease [94,95]. Is CD82 association with other tetraspanins critical for its suppressive function and does CD82 act to inhibit their activity? What are the metastasis "accelerators" that CD82 suppresses? Finally, are there functional links between loss of CD82 and loss of the other metastasis suppressor genes, and how many metastasis suppressor functions need to be removed to generate a metastatic cancer cell? All these questions remain largely unanswered, but progress is being made in all areas.

2.4. Cell-matrix adhesion

The most well characterized CD82 function in non-immune cells is its role in integrin-mediated cell migration on extracellular matrix. The logical connection to metastasis suppression is that enhanced integrin-mediated migration is crucial for detachment of tumor cells from their local microenvironment, followed by migration and penetration into the blood and lymph system. Consistent with this are numerous reports demonstrating that re-expression of CD82 in metastatic tumor cells inhibits in vitro migration and matrigel invasion [3,114,95a,5,95b,98,99]. Additionally, the integrin/CD82 associations discovered in immune cells were found to exist in non-immune cells. Immunofluorescent staining of MDA-MB-231 breast cancer cells adherent to a laminin matrix demonstrated colocalization of CD82 with several tetraspanins, CD9, CD63, CD81, and CD151, within α3β1-containing adhesion structures. CD82 was present in α3β1 immunoprecipitates, along with CD81 and CD151. Talin and MARCKS, known PKC substrates, were present in the adhesion structures containing the tetraspanins, but FAK and vinculin were not, consistent with a role for tetraspanins in regulating early adhesion/migration events [96]. CD82 has subsequently been reported to coimmunoprecipitate with α3β1, α6β1, and α5β1 in various adherent cells (Table 2) [29,49,97].

Reduced adhesion-induced signaling has been observed in CD82-re-expressing metastatic tumor cells. Changes in integrin-mediated signaling include reduced Src activation and phosphorylation of its downstream targets, i.e. p130Cas and Src-dependent FAK phosphorylation sites, as well as alterations in p130-Cas/CrkII complex formation [98,99]. Direct interference with these pathways reduced cell migration and invasion, as did CD82 re-expression. CD82 was shown to impact Rho GTPase signaling in immune cells; however, this has not been directly investigated in the CD82 re-expressing tumor cells [66]. Nonetheless, reduced signaling via p130Cas/CrkII would be predicted to inhibit Rac signaling. Strikingly, CD82 appears to have little or no effect on integrin-mediated activation of the Ras/Erk or

PI-3K/Akt signaling pathways, suggesting specificity with respect to the types of signaling pathways that CD82 impacts (Table 2).

One study demonstrated that PMA treatment of immune cells induced PKC association with CD82 as well as CD9, CD53, CD81, and CD151. CD81 and CD151 could be cross-linked to PKC indicating a direct association. Whether this was also true for CD82 was not mentioned [100]. PMA treatment also induced an association between PKCα and the extracellular domain of α3β1 integrin. It was subsequently shown that PKC can bind to the β1 integrin tail and regulate cell motility [101,102]. No follow-up studies in metastatic tumor cell lines re-expressing CD82 have been reported. However, the association between CD82 and the PKC substrates talin and MARCKS in adhesion structures [96] suggests there is likely to be an association, even if it may be indirect (Table 2). Furthermore, the role of PKC in regulating actin dynamics in cell migration is well documented [103]. PKC-mediated phosphorylation of several substrates, MARCKS, adducin, fascin, talin, and ERMs, reorganizes existing actin structures allowing for the reassembly of new actin structures that promote migration. It is possible that CD82 suppresses cell motility through a negative influence on PKC (Fig. 1).

In contrast to CD82, CD151 enhances and is required for cell migration. CD151 not only associates with PKC [100], but also associates with PI-4K [104]. PI(4,5)P$_2$ interacts with MARCKS and is involved in recruiting actin remodeling proteins upon dissociation of MARCKS following PKC-mediated phosphorylation [103]. Thus, in the absence of CD82, CD151 could drive cell migration by coordinating PKC and PI-4K signaling to drive actin reorganization. When CD82 is present it may sequester PKC away from CD151 and limit its ability to stimulate cell migration.

Another model for CD82-mediated inhibition of cell motility is provided by studies on tetraspanin-associated proteins EWI-2 and EWI-F. The single-pass Ig superfamily membrane proteins EWI-2 and EWI-F were originally identified as CD9- and CD81-associated proteins in chemical cross-linking studies [105–108]. Subsequently, EWI-2 was found in close association with CD82 in a complex with CD81 [109](Table 2). Over expression of EWI-2 in metastatic prostate cancer cells inhibited cell migration and further synergized with CD82 [109]. Over expression of EWI-2 in A431 cells, which do not have CD82 [110], also suppressed their migration [111]. EWI-2 enhanced α3β1/CD81 complex formation in A431 cells, suggesting that the α3β1/CD81complex is inhibitory to migration. The EWI-2 cytoplasmic domain was required for its inhibitory function. Pulldown experiments demonstrated a direct interaction between ERMs and the cytoplasmic domain of EWIs. The exact amino acids involved in this interaction were not determined, but the highly charged basic residues maybe involved. A dominant negative form of moesin delocalized EWI. Inhibition of EWI-2 expression by siRNA stimulated cell migration and increased phosphorylation of ERMs [112]. Thus CD82 may simultaneously function to inhibit PKC activity, while also controlling localization of EWI-2 protein, ultimately limiting actin remodeling events and migration by controlling ERMs (Fig. 1).

2.4.1. Integrin trafficking

PKCα activation stimulates β1-dependent migration and induces β1 integrin internalization and endocytosis. PKCα stimulated migration is inhibited by blockade of endocytosis [101]. Several tetraspanins, including CD82, contain an endosomal sorting motif (Tyr-X-X-φ) in the C-terminal tail and tetraspanins are often found on internalized vesicles [51,113]. It is possible that tetraspanins influence PKC-mediated internalization of integrins. The reduced cell adhesion observed in some CD82 re-expressing cells could be due to reduced cell surface integrin expression due to changes in integrin internalization [6,29,114,115]. For instance in one study, CD82 re-expression in a metastatic prostate cancer cell line reduced α6 integrin surface levels, reduced cell adhesion, and enhanced α6 internalization [97]. This result is somewhat unexpected since increased internalization would

Table 2
CD82-associated and effector proteins

Extracellular
DARC/duffy antigen
Gangliosides: GD1a, GM2, GM3
Membrane associated
Tetraspanins: CD9, CD81, CD151, CD63
Integrins: α3β1, α6β1, α5β1, α4β1
Type I TM: EWI-2, GGT, MHC II
Receptors: EGFR, c-Met, TCR
Type III TM: VangL1
Intracellular
PKC
Src
Rho GTPases
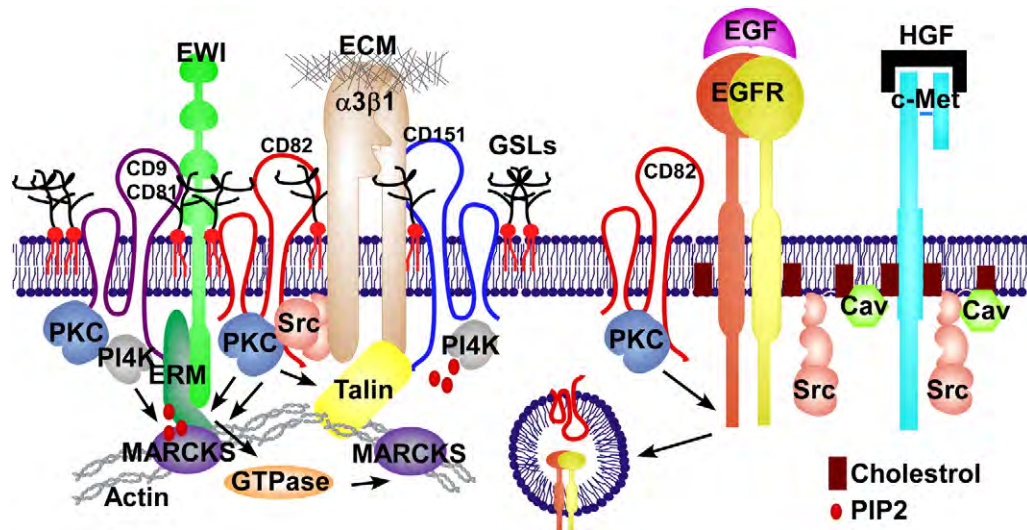
Fig. 1. Model for CD82 regulation of integrin and receptor tyrosine kinase signaling. Integrin Signaling: CD82 in association with other tetraspanins (CD9, CD81, CD151) within the ganglioside (GSLs) enriched microdomains control the signaling output from integrins (α3β1) and associated proteins (EWI) to tightly control early cytoskeletal rearrangements associated with integrin engagement with the matrix (ECM). Talin association with integrin tails and ERM association with EWI are both targets of PKC signaling, which affects their ability to bind actin and control actin polymerization. The actin binding protein MARCKS is targeted by PKC as well as Rho GTPases. Src also controls actin dynamics. In addition, actin polymerization requires the production of PIP2 (red spots) via PI-4K, which binds both ERM and MARCKS. It is not know whether CD82 association with PKC enhances or inhibits its activity within the complex, but CD82 has been shown to inhibit Src. RTK Signaling: The ability of CD82 to inhibit EGFR signaling by its ligand EGF within caveolin (Cav) cholesterol-rich lipid rafts (brown bars) is mediated by PKC-dependent phosphorylation of EGFR cytoplasmic tail and its subsequent internalization. Whether the same mechanism is true for regulating HGF-induced c-Met activity is unknown.

be expected to increase cell migration, when in fact CD82 suppresses migration in these cells [98]. However, it was not clear whether the internalization studies were done with cells plated on the relevant matrix. The total cellular levels of α6 integrin were unchanged, indicating that CD82-mediated internalization does not lead to degradation of the integrin. The effect of CD82 on internalization of other integrins was not monitored.

The cell surface expression of α3 or α6 integrins was not altered in CD151 null cells generated from mutant mice [90] or by siRNA-knock down [116,117]. However, the internalization rate of α3β1 in these cells was reduced, which is consistent with reduced migration due to reduced integrin turnover. Mutation of the sorting motif in the C-terminal cytoplasmic domain markedly attenuated CD151 internalization and blocked migration; however, only subtle changes were observed in integrin internalization [118]. It is possible that the ability of the CD151/α3β1 complex to associate with other tetraspanins, which also have internalization motifs, may be responsible for facilitating efficient α3β1 internalization. Alternatively, inhibition of cell migration involves additional changes. A direct effect of the CD82 sorting motif on integrin internalization has not been reported.

Does CD82 influence integrin synthesis and/or presentation at the cell surface? Other tetraspanins have been shown to regulate early biosynthesis of their associated molecules. Examples include CD151/α3β1 [119,120], uroplakins/UPs [121], CD81/CD19 [122], and CD9/pre-β1 integrin [123]. In one report, re-expression of CD82 in tumor cells negatively influenced pre-β1 integrin processing resulting in reduced β1 integrin on the cell surface [124]. Tetraspanins may influence integrin trafficking; however, currently the link to CD82-mediated suppression of metastasis is weak.

2.4.2. Integrins vs tetraspanins

Despite extensive evidence that CD82 inhibits integrin-dependent signaling and migration, the evidence that CD82 suppresses metastasis via this mechanism is lacking. An analysis of the domains within CD82 that are responsible for 1) interaction with integrins, 2) suppressing integrin-mediated migration/invasion, and 3) metastasis suppression is required. While CD82 can colocalize and be coimmunoprecipitated with several different integrins, this interaction appears to be indirect since it is only observed in mild detergents.

A direct interaction between the α3 and α6 integrins and CD151 was demonstrated by their ability to be chemically cross-linked, stability of the association in harsh detergents, and identification of the interacting domains by mutagenesis [119,120,125]. CD151 interacts with the extracellular stalk domain of α3 (aa 570–705), a region not directly involved in mediating cell adhesion [125]. The integrin-binding region of CD151 mapped to an 11 amino acid region within the EC2 domain (aa 195–205) containing the sequence QRD (aa 194–196) [119,120]. Mutation of the α3 binding region in CD151 had no affect on the ability of CD151 to associate with itself or other tetraspanins (CD9, CD81) [120]. However, CD151 containing a QRD mutation disrupted α3/α6-dependent formation of cellular cables on Matrigel and altered cell spreading [119]. In fact, removal of CD151 from cells disrupts α3/α6 association with other tetraspanins and impairs integrin internalization [117,126]. Thus any effects CD82 may have on α3 or α6 integrin-mediated events are likely to involve interactions with CD151.

CD82, like all tetraspanins thus far investigated, is palmitoylated [50,127–130]. Palmitoylation at membrane proximal cysteines promotes protein–protein interactions between weakly associated proteins and is important for lipid raft association of signaling molecules [131]. In fact, inhibition of tetraspanin palmitoylation significantly reduces tetraspanin–tetraspanin interactions [129,132]. Mutation of all five membrane proximal cysteines in CD82 abolished palmitoylation, reduced but did not abolish association with CD9 and CD81, and failed to suppress migration and invasion of tumor cells. The CD82 palmitoylation mutant also no longer suppressed p130Cas-CrkII signaling [50]. However, the effect of palmitoylation loss on association with integrins or metastasis was not reported.

The integrins that specifically associate with tetraspanins, namely α3, α6 and β4, are also palmitoylated. Mutation of the 7 potential palmitoylation sites in β4 integrin impaired cell spreading and reduced signaling to p130Cas. β4 association with tetraspanin complexes was reduced upon loss of β4 palmitoylation [133]. Although the ability of CD82 to suppress integrin-mediated events is dependent upon its palmitoylation, it is not clear whether it is the association with other tetraspanins or integrins or both that is important.

Palmitoylation promotes the association of proteins with lipid rafts. However, the evidence that tetraspanins associate with classical

cholesterol-rich lipid rafts is limited. Instead, tetraspanins are most commonly associated with ganglioside-enriched membrane microdomains (GEM or TEM). This observation has been made primarily in "resting" cells, i.e. cells in which specific signaling pathways have not been stimulated. However, in immune cells, co-crosslinking of BCR and CD19 causes BCR, CD19, and CD81 to appear in cholesterol-rich lipid rafts, which is required for Vav and PLCγ recruitment into the rafts [134]. Furthermore, the level of palmitoylation was increased specifically in lipid raft-associated CD81. Blockade of palmitoylation prevented CD19/CD81 association with rafts. Thus, under some stimulatory conditions there may be significant shifting of tetraspanins from GEM/TEMs into rafts, especially during active signaling processes. These findings also support the idea that palmitoylation of tetraspanins may be regulated, as has been observed for other molecules [131].

A class of 23 enzymes named for the Asp–His–His–Cys active site motif (DHHC), are involved in the transfer of palmitoylate to target cysteine residues. DHHC2, 5, 7 and 11 were identified in tetraspanin complexes by mass spec. Of these, DHHC2 was most efficient at stimulating palmitoylation of CD9 and CD151. DHHC2-dependent palmitoylation promoted interactions between CD9 and CD151, but had no affect on integrin β4 palmitoylation or association of α3 integrin with CD151. Mutation of the active DHHC motif blocked tetraspanin palmitoylation. Furthermore, loss of DHHC2, but not 6 other DHHC proteins, not only diminished CD9 and CD151 palmitoylation, but also dramatically enhanced their degradation. Loss of DHHC2 reduced cell–cell contact, probably as a result of general loss of tetraspanins due to degradation [135].

Could tetraspanin interactions influence the function of each other? CD82 appears to regulate cell functions that other tetraspanins enhance. For instance, CD151 and CO-029 have the opposite effects on cell migration as CD82 and both are over expressed in cancers. Blocking CD151 function with antibodies or inhibiting expression with siRNA produces cell migration and invasion, suppresses signaling to FAK, Rac1, and Src kinases, and delays tumor progression in mice [117,126,136]. CO-029 enhances cell migration through its association with α6β4 and CD151 in invasive cancer cells [136a,136b]. CD9 generally has the similar suppressive effects as CD82, but this is dependent on cell type. Recently Net-6 (Tspan13) was demonstrated to behave as a tumor suppressor [137]. The apparently opposing actions of different tetraspanins, suggests that CD82 re-expression in tumor cells may restore the tetraspanin "balance" to specifically impose tighter regulatory controls on integrin-mediated motility. The converse of that, i.e. loss of CD82, would cause an "imbalance" in tetraspanins that favors enhanced motility. More rigorous experimentation is required to sort this out.

2.5. Growth factor receptor signaling

Are integrins the only molecules that CD82 regulate? Tetraspanins play a critical role in regulating receptor tyrosine kinase signaling in immune cells, i.e. TCR and BCR [66,134]. In addition, signaling through the receptor tyrosine kinase c-Kit is inhibited when it associates with tetraspanins in myeloid cells [138]. CD82 has been shown to affect signaling mediated by at least 2 receptor kinases present in non-immune cells, EGFR and c-Met.

2.5.1. EGFR and c-Met

In a normal mammary epithelial cell line, HB2, CD82 expression reduced EGF-stimulated wound migration, EGFR and Shc tyrosine phosphorylation, and Grb2 association with EGFR [132]. CD82 expression increased EGF-induced EGFR internalization. CD9 was shown to have a similar effect on EGFR in several tumor cell lines [139]. EGFR complexes containing CD9 or CD82 could be detected, but integrins were not present in these complexes.

Re-expression of CD82 in metastatic prostate cancer cell lines reduced both ligand-dependent and -independent activation of the HGF/SF receptor c-Met, and suppressed c-Met-dependent invasion [99]. c-Met signaling and invasion could be rescued at high concentrations of HGF, suggesting that CD82 modulates the level of c-Met activation rather than completely suppressing it. c-Met could not be detected in CD82 complexes, nor did it significantly colocalize with CD82 in cells, suggesting an indirect mechanism of regulation. Similarly, HGF-induced cell migration as well as Grb2 and p85 association with c-Met was reduced upon CD82 re-expression in H1229 lung carcinoma cells [140]. In contrast, removal of CD151 by siRNA in salivary gland adenoid cystic carcinoma tumor cells severely reduced HGF-stimulated cell migration, while CD151 over expression increased c-Met-mediated migration [141].

2.5.2. Gangliosides

Glycosphingolipids (GSLs) are associated with organized membrane microdomains. Gangliosides in particular modulate 1) growth factor-stimulated receptor tyrosine kinases, 2) integrins complexed with tetraspanins, and 3) downstream signaling molecules such as Src and small G proteins [142]. The ability of tetraspanins, and in particular CD82, to modulate RTK and integrin signaling may be mediated by gangliosides, which are found within the tetraspanin-enriched microdomains [143].

The ability of CD9 to promote tumor cell motility is suppressed by the exogenous addition of GM3 ganglioside. This is specific, in that addition of GM1 did not have a similar effect. IdID mutant CHO cells that are defective in UDP-Gal 4-epimerase, cannot synthesize gangliosides unless cultured in the presence of galactose. Expression of CD9 promotes motility in the absence of galactose, which is suppressed when galactose is present [144]. CD9/α3 integrin complexes could be detected by co-immunoprecipitation only when GM3 was present [145]. A similar relationship was observed in human bladder cancer cell lines; cells with higher GM3 levels are much less invasive than those with lower levels. A stronger interaction between α3 integrin and CD9 was detected in high GM3 expressing cells. The non-invasive cells could be converted to invasive cells by depletion of GM3 or siRNA knockdown of CD9 and vice versa. GM3 addition induced Csk translocation into GEMs and inhibited Src activation [146]. Interestingly, v-Jun-induced cell transformation greatly reduces the levels of GM3 and GM3 synthase mRNA in fibroblast cell cultures. Re-expression of GM3 synthase suppressed growth in agar and increased the levels of CD9/α5β1 complexes in those cells [147]. Thus suppression of migration is mediated by enhanced complex formation between GM3/CD9/integrins, suggesting this complex limits migration by diminishing integrin function.

The level of GD1a ganglioside increased and colocalized with CD82 on the cell surface when CD82 was re-expressed in normal mammary cells [148]. Specific removal of GD1a decreased CD82 association with tetraspanin CD151, but increased CD82 association with EGFR [149]. Unfortunately, the effect of specifically depleting GD1a on EGFR signaling was not determined. In a separate study CD82-induced suppression of EGFR phosphorylation and internalization was shown to be dependent on an association between PKCα and CD82 and the lipid raft protein caveolin-1. In this model EGF-induced EGFR internalization is regulated by PKCα-mediated phosphorylation of EGFR at Thr654. The ability of the caveolin-1/CD82/PKCα complex to inhibit EGFR signaling was dependent on GM3 [150]. Thus "mixing" between membrane microdomains, i.e. caveolin-containing lipid rafts and GEM/TEMs, may occur subsequent to activated signaling and function to limit the signaling response (Fig. 1).

GSLs impact c-Met signaling as well. Re-expression of CD82 in an invasive bladder cancer cell line reduced c-Met signaling in response to low doses of HGF and inhibited HGF-dependent motility. Removal of GM2 prevented CD82-mediated suppression of c-Met activation and motility. GM2, but GM3, could be detected in a complex with CD82 [151]. The level of ganglioside expression clearly has an impact on the ability of tetraspanins to regulate RTK and integrin signaling.

Together the integrin and growth factor receptor studies suggest that CD82 functions to suppress cell migration through the dynamic assembly and disassembly of carbohydrate-enriched complexes consisting of tetraspanins and integrins within GEMs/TEMs. These interactions are critical for regulating signal transduction pathways that impact cell migration via integrins. In addition, CD82 can negatively impact RTK signaling within in lipid rafts by limiting signaling and increasing internalization. There is specificity in that CD82 exerts its suppressive effects on migration and RTKs primarily by targeting Rho, PKC, and Src signaling pathways and has a minimal impact on proliferation-regulated pathways (Fig. 1). This fits well with the known affects of CD82 loss on tumors in vivo.

2.5.3. GPCR

Secreted tissue transglutaminase (TG2) is a Ca^{2+}-activated extracellular matrix protein involved in matrix cross-linking, which binds fibronectin and integrins, and stimulates cell adhesion, spreading, and focal contact assembly [152]. TG2 levels are decreased in aggressive tumors and metastases [153]. Expression of GPR56, recently identified as the TG2 receptor [154], is dramatically reduced in highly metastatic cells [154,155]. Over expression of GPR56 suppresses tumor growth and metastasis, while reduced expression enhances tumorigenesis and metastasis.

GRP56 has homology with the LNB-TM7 subfamily of GPCRs. The N-terminus of these molecules contains adhesion molecule domains. The planar cell polarity cadherin-like protein, Flamingo (Celsr1), belongs to this class of GPCRs [156]. GPR56 is highly expressed in glial cells, particularly in regions that contact the basement membrane. Genetic mutations in GPR56 cause congenital brain malformation in humans, and GPR56 is required to maintain basement membrane integrity during cortical development [157].

GPR56 was also identified as a CD81-associated protein in retinoic acid-differentiated NT2 teratocarcinoma cells by mass spectrometry. GRP56 and the heterotrimeric G protein subunits, $G\alpha(q/11)$ and $G\beta$, associated with both CD9 and CD81, but not CD151 or CD63. CD81 antibody caused dissociation of $G\alpha(q/11)$ from GPR56/CD81, while PMA treatment induced GPR56 dissociation and internalization away from the CD81/ $G\alpha(q/11)$ complex [158]. Thus, depending on the stimulatory signal, CD81/CD9 may differentially regulate signaling through GPR56. The link between CD81 and GPR56 is interesting given the dramatic overproduction of glial cells during brain development in CD81-null mice. The defect in CD81 null and GPR56 mutant glial cells may be linked to the role of tetraspanins in trafficking. Human genetic mutations in GPR56 that map to the N-terminal domain result in poor surface expression due to reduced intracellular trafficking [159]. Reduced GPR56 surface expression would be expected to increase the proliferation of glial cells.

CD82 is not well expressed in the brain, but is present in many other tissues that develop tumors. Links between CD82 and GPR56 or other GPCRs have not been reported.

2.6. Invasion

Thus far, we have focused primarily on adhesion and migratory events regulated by integrins and tetraspanins. However, metastasis requires the activation of extracellular proteases that degrade the extracellular matrix to facilitate cell invasion and movement away from the primary tumor. Several major protease systems are known to be activated in invasive cancer cells and three have been linked to tetraspanins; a disintegrin and metalloprotease (ADAMS), urokinase plasminogen activator and receptor (uPA/uPAR), and matrix metalloproteases (MMPs).

2.6.1. ADAMS

ADAMs are transmembrane proteases whose activity is restricted to membrane localized substrates. One major function of ADAMs is the shedding of cell surface growth factor ligands such as HB-EGF and TGFα leading to paracrine and autocrine activation. The disintegrin and cysteine-rich domains on the extracellular domain of ADAMS also allows for interactions with integrins or syndecans to facilitate cleavage of the ECM [160,161]. CD9 is known to associate with HB-EGF and TGF-α and regulate their juxtacrine signaling activity [162,163]. GPCR signaling stimulated the association of HB-EGF with CD9 and enhanced cleavage of HB-EGF, which was dependent on ADAM10 [164]. Other studies suggest that ADAM17 is the primary enzyme involved HB-EGF and TGF-α shedding [165]. Association of ADAM17 with tetraspanins has not been reported. Fertilization studies indicate critical roles for CD9/CD81 and ADAM2 in egg-sperm interactions, such that loss of these molecules on eggs and sperm respectively inhibit fertilization [76,77,79,166,167]. However, the exact functional relationship between ADAMs and tetraspanins in sperm–egg fusion remain controversial. Despite demonstrated interactions between tetraspanins and ADAMs, the effect of tetraspanins on ADAM activity has not been determined. ADAMs are known to be involved in matrix degradation in metastatic lesions, but any links to tetraspanins in this context have not been reported.

2.6.2. uPA and uPAR

Binding of urokinase-type plasminogen activator (uPA) to its receptor (uPAR) locally cleaves plasminogen leading to generation of the diffusible protease plasmin. uPAR has been shown to associate with several integrins including $\alpha3\beta1$ and $\alpha5\beta1$. uPAR binds directly to the beta propeller of $\beta1$ integrins and affects many functional aspects of integrins during migration [168,169]. Thus given the close association between uPAR and integrins, and integrins and tetraspanins, it is highly likely that uPAR activity may be regulated by tetraspanins. CD82 expression in a normal mammary epithelial cell line, HB2, was shown to significantly reduce plasminogen activation. Reduced plasminogen activation was due to reduced uPA binding to uPAR in the presence of CD82, but was not mediated by an interaction between uPAR and CD82. Instead, CD82 expression led to a redistribution of uPAR to $\alpha5\beta1$ containing focal adhesions [170].

The role of CD82 in regulating uPAR activity in a metastatic tumor setting has not been investigated. However, it has recently been shown that uPAR activation in metastatic prostate cancer cells generates a cleaved form of $\alpha6$ integrin that promotes increased migration and invasion on laminin substrates [171]. Furthermore, c-Met activation via HGF has been shown to stimulate uPAR activity in MDCK cells and in metastatic prostate cancer cells [172,173]. If CD82 negatively regulates both c-Met and uPAR activity, then CD82 should suppress generation of the cleaved $\alpha6$ integrin in metastatic cells.

2.6.3. MMPs

A role for MMPs in tumor angiogenesis, tumor invasion, and establishment of metastases at secondary sites has been well established [174]. Antibody-mediated ligation of $\alpha3\beta1$-tetraspanin (CD9, CD63, CD151, CD81) complexes in MDA-MB-231 cells stimulated the production of MMP-2, but not MMP-9, and increased invasiveness [175]. Elevated CD9 expression in a melanoma cell line induced MMP-2, but suppressed MMP-9 expression [176]. Whereas expression of CD151 in the same cell line stimulated MMP-9 expression [177]. Elevated MMP-2 and MMP-9 expression was dependent on cell adhesion to laminin, signaling through Jnk, and lead to increased AP-1 binding on the two promoters. Re-expression of CD82 in H1299 lung carcinoma cells reduced MMP-9 activity, but elevated expression of the metalloprotease inhibitor TIMP1 [178].

Clearly there are functional relationships between tetraspanins and extracellular proteases. However, the mechanisms involved in tetraspanin-dependent regulation of their activity and the overall importance in metastasis suppression or promotion still needs to be determined.

2.7. Homotypic cell–cell interactions

An under-appreciated, but highly relevant, aspect of CD82 function is its role in cell–cell adhesion. Numerous studies have demonstrated that CD82 is localized at and induces cell–cell adhesion [178a,68,178b,115,114,95a]. The exact nature of the cell–cell interaction has not been deciphered, except the interaction is in some cases Ca^{+2}-independent [178b,114,95a]. Loss of the cell–cell adhesion molecule E-cadherin is highly associated with metastasis in epithelial cancers and is considered to be a metastasis suppressor. However, the Ca^{+2}-independence of CD82-mediated adhesion rules out E-cadherin as a direct target, suggesting that in addition to E-cadherin loss, metastasis may require disruption of additional cell–cell adhesion molecules that are regulated by CD82.

2.7.1. Claudins

One type of Ca^{+2}-independent cell adhesion molecule that could be regulated by CD82 is claudins, which form tight junctions in polarized cells. A few family members are known metastasis suppressors. Tumorigenic changes in cellular localization and expression of claudins have been reported [179], although exactly which claudin is changed in which cancer type remains controversial. While CD82 itself has not been directly implicated, several examples of direct associations between tetraspanins and claudins have been reported.

In a yeast two hybrid screen, the C-terminal half of tetraspanin OAP1/Tspan3 was found to interact with claudin11; which forms tight junctions in myelin sheaths of the CNS. OAP1, claudin-11, and β1 integrin formed a complex in oligodendrocytes. Claudin-11/OAP1 interactions were involved in promoting proliferation and cell migration of oligoendrocytes in vitro [180]. Claudin-7, EpCAM (another adhesion molecule), tetraspanin CO-029, and CD44v6 form a complex in gastrointestinal tumors. Co-expression of these 4 markers is inversely correlated with disease-free survival in human colon cancer. Claudin-7 was required for EpCAM to associate with CO-029 and CD44v6 [181]. Claudin-1 was shown to directly interact with CD9 based on chemical cross-linking studies in non-polarized A431 cells. Claudin-1 also interacted with CD81 and CD151. Interestingly in polarized epithelial cells, CD9/claudin 1 complexes were not detected [182]. It should be noted that the claudin studies involving tumor cells primarily focused on tetraspanins known to potentiate tumor aggressiveness, i.e. CO-029, CD9, CD151, and suggest that these tetraspanin associations with claudins in tumor cells may act to inhibit tight junction formation. Most of these tumor cells do not express CD82, and most normal polarized epithelial cells do. One possibility is that re-expression of CD82 could shift tetraspanin/claudin interactions to restore cell–cell adhesion (Fig. 2A).

2.7.2. E-cadherin

CD82 re-expression in h1299 non-small cell lung carcinoma cells increased homotypic cell–cell aggregation, which was dependent on calcium and blocked by anti-E-cadherin antibody. Surprisingly, CD82 expression did not alter E-cadherin distribution, which was present at the cell surface at cell–cell junctions. However, β-catenin distribution was altered [183]. Over expression of CD151 in A431 cells also enhanced E-cadherin-based cell–cell adhesion. cdc42 and Rac were elevated in the CD151-overexpressing cells and CD151-induced cell–cell adhesion was dependent on PKC [184]. Conversely, immortalized epithelial cells taken from mice lacking α3 integrin or treated with CD151 siRNA displayed reduced cell–cell adhesion. Loss of α-actinin association with E-cadherin, increased β-catenin tyrosine phosphorylation, and increased stress fibers (indicative of elevated Rho activity) were also seen in α3 null and CD151 siRNA cells [185]. However, E-cadherin along with β-catenin remained associated with the cell membrane. Thus tetraspanins can regulate E-cadherin dependent adhesion, not by regulating E-cadherin directly, but by regulating the
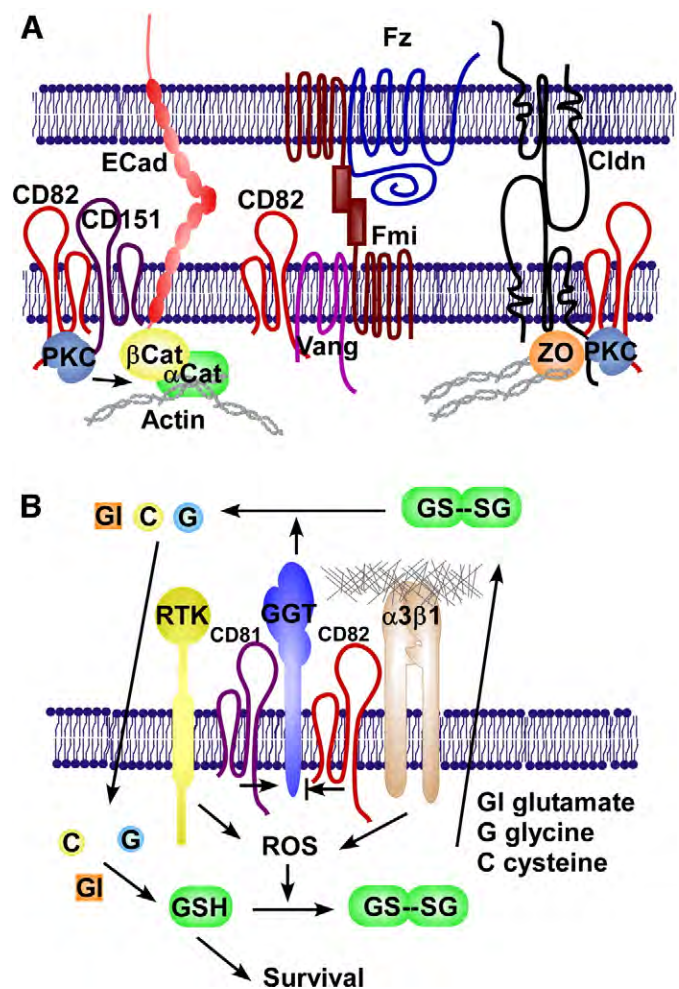
Fig. 2. A) Model for CD82 regulation of homotypic cell–cell interactions. The ability of CD82 and other tetraspanins (CD151) to affect adherins junction E-cadherin (ECad) mediated cell–cell interactions is regulated at the level α-catenin (αCat) and β-catenin (βCat) interactions with the actin cytoskeleton. PKC is involved in regulating those interactions. CD82 can also directly interact with VangL1 (Vang), which is involved in establishing cell polarity through interactions with the atypical cadherin Flamingo (Fmi) in one cell and Flamingo and the Wnt receptor Frizzled (Fz) in the adjoining cell. The effect of CD82 on this interaction is unknown. Tetraspanins can also associate with claudins (Cldn), which are normally linked to the actin cytoskeleton via ZO proteins at tight junctions. PKC signaling regulates ZO activity. Whether CD82 controls PKC signaling at tight junctions has not been determined. B) Model for CD82 regulation of oxidative stress. CD81 and CD82 both associate with the transmembrane γ-glutamyl transpeptidase (GGT), which catalyzes the first step in the degradation of GSH and GSH-conjugates (GS–SG) into glutamate (Gl), cysteine (C) and glycine (G). These are transported back into the cell to regenerate GSH. GSH is responsible for reducing ROS generated by local signaling through RTKs or integrins (α3β1). The correct balance ensures cell survival. The effect of specific tetraspanins on GGT activity is unknown.

association of the E-cadherin/β-catenin complex with the cytoskeleton to stabilize cell–cell adhesion. Surprisingly, CD151 and CD82 behave the same way, i.e. both increase cell–cell adhesion. However, the consequences of increased cell–cell adhesion may be different as a result of specific changes in downstream signaling pathways.

The downstream signaling pathways required for CD82-mediated E-cadherin adhesion have not been investigated. However, the molecules involved in CD151-induced cell adhesion have been. CD151 and α3β1 integrin were both required for efficient expression of PTPμ, a transmembrane protein tyrosine phosphatase involved in cadherin-mediated adhesion. In addition, E-cadherin and β-catenin could be detected in an α3β1/CD151 complex containing PTPμ, PKCβII, and RACK1. Loss of α3 disrupted these interactions. The stalk domain of α3 integrin, required for interaction with CD151, was sufficient to

rescue PTPμ expression, restored the multi-complex, and increased cell–cell adhesion in α3 null cells. These data suggest a strong requirement for α3β1 in cell–cell adhesion. In fact, it was demonstrated that α3β1 within the cadherin–catenin complex was distinct from that involved in adhesion to laminin. What will be most valuable is to determine how CD82 impacts these interactions (Fig. 2A).

2.7.3. PCP and Wnt

Vangl1 was pulled out of a two hybrid screen using a C-terminal fragment of CD82. Loss of Vangl1 in CT-26 colon cancer cells reduced, while over expression enhanced, adhesion and invasion. Vangl1 over expression did not increase tumorigenesis or metastasis, but loss of Vangl1 decreased growth and inhibited metastasis. Vangl1 levels were higher in human gastric tumors and metastases, compared to normal tissues [186]. These data suggest that Vangl1 promotes a more metastatic phenotype. Over expression of Vangl1 reversed the suppressive effects of CD82 on matrigel invasion, suggesting that Vangl1 is downstream of CD82.

The most well characterized role for Vang-like proteins (aka strabismus) are their involvement in establishing planar cell polarity (PCP) in epithelia during eye and wing development in Drosophila [187]. Strabismus functions in the context of Frizzled (a Wnt receptor) to communicate cell polarity. Flamingo, an atypical cadherin of the LNB-TM7 subfamily of GPCRs, functions to establish an asymmetric cell–cell interaction by causing recruitment of a Frizzled complex containing Disheveled and Diego at the cell–cell interface in one cell and a Strabismus/Prickle complex at the same cell–cell interface in the other cell [187,188]. Exactly how Flamingo establishes the asymmetry is not clear, but may involve two functional forms of Flamingo on either side of the membrane (Fig. 2A).

Mammals have two strabismus-like proteins, Vangl1 and Vangl2, as well as mammalian homologs of the other PCP proteins. Mice lacking Vangl1 are viable and fertile, but do display subtle alterations in polarity of inner hair cells of the cochlea, a PCP-dependent process. The Vangl2 null mice are embryonic lethal with defects in neural tube closure. Double heterozygotes have developmental defects in the neural tube, inner ear, and heart [189]. The mild phenotype in Vangl1 null mice suggests Vangl1 may have cell–cell functions independent of PCP. Thus Vangl1 is well poised to function as a CD82-regulated target to control cell–cell interactions.

Intestinal trefoil factor (ITF) is a secreted factor that promotes the migration of intestinal epithelial cells during mucosal repair. Vangl1 is Ser/Thr phosphorylated in response to ITF and removed from the cell membrane following ITF stimulation. Loss of Vangl1 inhibited ITF-dependent migration [190]. This function of Vangl1 is consistent with its role as a pro-metastatic factor; however, the HT29 cells in which Vangl1 function was assessed, express CD82 [191]. The internalization and removal of Vangl1 from the membrane would be consistent with a role for CD82 in regulating trafficking of Vangl1. The exact relationship between CD82 and Vangl1 needs to be better characterized.

An extracellular p90K Vangl1-interacting protein was detected in a two hybrid screen [192]. p90K is a soluble oligiomerized ligand for galectin receptors. It is a strong inducer of ICAM-1 and VCAM-1 expression on tumor endothelium [193]. Both ligand and receptor are strongly up-regulated in tumors and associated with metastasis and poor prognosis. Interestingly, p90K can mediate homotypic cell–cell adhesion via galectins on adjacent tumor cells and can enhance cell matrix adhesion. These processes may be critical for cancer cell survival in the bloodstream and during the establishment of metastatic colonies [194]. Thus p90K/galectin has the potential to play a role in many steps in tumor progression and metastasis. However, the connection between p90K, Vangl1, and CD82 remain puzzling. If Vangl1 acts to promote metastasis [186], then it would be expected to enhance p90k secretion and that CD82, acting as a metastasis suppressor, would suppress p90K. However, loss of Vangl1 led to increased p90k secretion [192]. The effect of CD82 on p90K was not investigated.

A second protein detected in the same two hybrid screen, protein kinase C interacting protein (PKCI), binds the intracellular domain of Vangl1 [192]. PKCI/Hint1, a histidine triad protein, interacts with pontin and reptin, which together inhibit β-catenin transcriptional activity [195]. This could explain the putative tumor suppressor activity of PKCI. These findings are intriguing with regard to the role of the reptin/β-catenin complex in expression of CD82 itself. Activation of the reptin/β-catenin complex, due to Wnt activation, suppresses CD82 expression in tumor cells, and the presence of PKCI could potentially prevent CD82 loss. The effects of Vangl1 and CD82 on PKCI function needs to be further evaluated.

2.8. Heterotypic cell–cell interactions

Entry into and out of the blood or lymphatic system requires physical interactions between the tumor cells and the endothelial cells. Thus in addition to mediating cell–cell interactions between tumor cells, CD82 could also impact heterotypic cell–cell interactions.

Several endothelial tetraspanins, including CD9, CD81, and CD151, were found to localize at tumor cell-endothelial cell junctions in both 2D and 3D cultures. Anti-CD9 antibodies specifically inhibited transendothelial migration of melanoma cells [196]. The specific molecules involved in cell–cell interactions were not identified. Tumor-specific expression of α4β1 integrin and binding to VCAM on endothelial cells, a process normally used by activated lymphocytes to migrate to distant tissues, is one possible mechanism. α4β1 integrin can be found on tumor cells, and is known to be regulated by tetraspanins. Whether tetraspanins are required in both cell types for these interactions still needs to be addressed. A recent study in CD151 null mice demonstrated the potential necessity of endothelial-specific CD151 expression for efficient extravasation of tumor cells from the blood stream (Martin Hemler, personal communication). However, blocking CD151 function in tumor cells themselves inhibits the initial dissemination of cells from the primary tumor, but has no effect on entry or exit from the blood stream [136].

The role of CD82 in the specific steps of metastasis has yet to be completely defined. However, tail vein injection of tumor cells re-expressing CD82 does not affect the ability of CD82 to suppress metastasis [5,9], suggesting that CD82's suppressive effects are not limited to events within the primary tumor. Re-expression of CD82 in HT1080 cells or loss of the CD82-specific E3 ubiquitin ligase gp78, which leads to enhanced CD82 expression, did not alter the ability of tumor cells injected via the tail vein to arrive in the lungs, i.e. CD82 had no effect on extravasation [9].

The blood group Duffy antigen (gp-Fy)/receptor for chemokines (DARC) is primarily expressed on the surface of erythrocytes and endothelial cells. DARC is a highly promiscuous chemokine receptor that binds a subset of chemokines involved in pro-angiogenesis, specifically CXCL1-5, CXCL7-11, and CCL2, CCL5, CCL7, and CCL17 [197]. However, chemokine binding does not transduce signals. It has been proposed that DARC serves either as a sink for excess chemokines or in some way regulates chemokine presentation or delivery. Genetic studies in mice suggest DARC exerts a negative effect on pro-angiogenic chemokine function. Transgenic mice over expressing DARC in the endothelium display a reduced angiogenic response in the corneal micropocket assay [198]. Conversely, DARC-deficient mice display enhanced angiogenesis in the matrigel plug assay [197]. Prostate tumors produced in DARC null mice had higher levels of angiogenic chemokines, increased tumor vessel density, and greatly augmented prostate tumor growth compared to tumors in wild type mice [199].

In a yeast two hybrid screen DARC was recently shown to interact with full length CD82. Re-expression of CD82 in tumor cells facilitated their adhesion to DARC positive cells. Adhesion of CD82 positive

tumor cells to DARC positive cells inhibited cell proliferation, reduced cell survival and induced expression of senescence markers TBX2 and p21 in the tumor cells. Furthermore, the ability of CD82 to suppress metastasis was significantly compromised in DARC null mice compared to wild type mice [200]. These findings suggest that CD82 suppresses metastasis by limiting tumor cell viability when cells are shed into the blood stream through interactions with DARC antigen on endothelial and blood cells. If this is true then, this is the first metastasis suppressor shown to act at this specific step in metastasis.

However, there are still several questions to be addressed regarding the putative interaction between CD82 on tumor cells and DARC on endothelial cells. First, it has not been demonstrated that the interaction between CD82 and DARC on these two cell types is direct. CD82 expression alone increases cell–cell interactions between CD82 expressing cells, and endothelial cells express ample CD82. The nature of the CD82-induced cell–cell interaction has not been determined and is not likely to be solely mediated by DARC, since CD82 expression stimulates cell–cell association between cells not expressing DARC. Second, the nature of growth suppressive/senescence signal that is transduced to the tumor cells is unknown. Is this a universal mechanism for cell clearance? If so, why are endothelial cells (which express CD82) not induced to die when then come in contact with red blood cells expressing DARC? Third, it is possible that interactions between CD82 positive tumor cells and endothelial cells also induce endothelial cell death, which could suppress metastasis by limiting efficient endothelial cell recruitment. Finally, can the failure of CD82 to suppress metastasis in DARC null mice be attributed to DARC's ability to modulate chemokine levels rather than a direct effect on tumor cell survival? Chemokines have been implicated in tumor cell homing and survival during metastasis. It is possible that the elevated levels of chemokines that would be expected to occur in the DARC null mice are sufficient to enhance the tumor survival or microenvironment such that metastasis is favored despite the presence of CD82.

Nonetheless, the putative role of CD82/DARC interactions in tumor metastasis is intriguing, and may be important in African-Americans. In addition to its role as a chemokine sink, DARC is the erythrocyte receptor for the malarial parasite. Approximately 70% of African-Americans lack erythrocyte-specific expression of DARC as protection against malaria infection. Coincidently, African-American men have a 60% greater incidence of prostate cancer, which develops as a more aggressive disease (i.e. metastatic) and at younger ages [199]. Thus the putative role of DARC in cancer progression and metastasis should not be overlooked, and that CD82 might be part of the mechanism warrants more intense investigation.

2.9. Survival

An important strategy for successful metastasis is acquiring the ability to survive in multiple environments. Loss of cell adhesion to the ECM is known to induce cell death. Tumor cell detachment and movement away from the primary tumor and transit through the blood stream requires integrin-independent survival. One potential result of CD82 loss would be to enhance tumor survival, through regulation of integrins or other cell surface proteins. CD82, along with tetraspanins CD9, CD53 and Net-6, have all been reported to regulate cell survival.

High levels of CD82 or CD9 expression in CHO cells under conditions that efficiently glycosylate CD82, promoted massive cell death after 11 day s in culture [201]. Cells incapable of glycosylating CD82 did not die, indicating that CD82-induced cell death requires glycosylation—which primarily occurs in the EC2 domain. However, in another study, a C-terminal deletion mutant lacking both the cytoplasmic tail and the EC2 domain was still capable of inducing apoptosis in HeLa cells [202]. The relative expression levels and cell surface expression of these mutants was not reported. It is important that the structure of tetraspanins be preserved for assessing full

functionality. For instance, multiple myeloma cell lines over expressing GFP-N-terminal CD82 or CD81, but not C-terminally fused proteins, underwent apoptosis [203]. In fact, the N-terminal tagged constructs failed to express properly at the cell surface. This may have triggered a misfolded protein stress response unrelated to CD82 function. Chimeric tetraspanin molecules, where different domains have been swapped between different tetraspanin molecules, have been used effectively in the past to assess the function of specific tetraspanin domains [107,108,122,204,205].

The tetraspanin Net-6 (Tspan13) is a newly described tumor suppressor gene whose expression level is lowest in highly aggressive breast cancers. Ectopic expression of GFP-NET-6 in MDA-MB-231 partially suppressed proliferative activity in vivo and in vitro, which resulted from an increase in apoptosis [137]. Deletion of Bap31, an ER membrane protein that regulates the export of integral membrane proteins out of the ER, results in decreased cell surface expression of CD9 and CD81. Bap31 did not influence cell surface expression of $\alpha5\beta1$ or $\alpha v\beta3$, but these integrins were not able to maintain ECM adhesion in the absence of growth factors. Subsequently, Bap31 null cells underwent apoptosis. Thus physical separation of tetraspanins from integrins profoundly affects integrin function. It remains to be determined if loss of Bap31 also affects surface expression of other proteins important in tetraspanin/integrin association and whether surface expression of tetraspanins is sufficient to restore integrin function [206].

A few studies suggest some possible mechanisms for tetraspanin-induced death. A monoclonal antibody to CD9 induced TUNEL and annexin-V staining in MKN-28 tumor cells. Apoptotic death was due to antibody-induced tyrosine phosphorylation of p46 Shc, which enhanced JNK and p38MAPK signaling and activated caspases [207]. CD82 was identified in a screen for apoptosis-inducing genes. Forty-two hours after transient transfection of several tumor cell lines (HEK293, PC3, HeLa; MCF7), a 40% increase in the sub G1 population was detected. In these cells CD82 promoted the generation of reactive oxygen intermediates, which upon blockade reversed the apoptotic phenotype. CD82 caused release of the intracellular glutathione (GSH) into the medium and apoptosis could be prevented by the addition of exogenous membrane-permeable GSH. CD82-induced GSH release was mediated by a CD82-dependent increase in Cdc42 activity. Blocking cdc42 activity reversed GSH release and apoptosis [202]. In contrast to CD82, CD53 is dramatically up-regulated in response to radiation exposure and appears to enhance cell survival by inhibiting the intrinsic apoptosis pathway [208]. Expression of CD53 leads to increased intracellular levels of GSH [209].

The ability of CD82 and CD53 to regulate GSH levels and affect cell survival may be related to their association with γ-glutamyl transpeptidase (GGT) [210]. GGT is a membrane protein involved in the extracellular degradation of GSH-conjugates and recycling of extracellular glutathione back into the cell. GGT breaks down extracellular GSH into glutamate and cysteinyl-glycine. Cysteine and glycine are released by a constitutive dipeptidase and all three are transported back into the cell for resynthesis of GSH via γ-glutamylcysteine synthetase (GCS) and GSH synthetase [211,212]. Extracellular GSH can arise from three possible sources in the body: GSH ingested as part of the diet, intracellular GSH transported out of the cell and into the extracellular environment or blood, and GSH secreted into lumens and extracellular spaces by secretory epithelia, such as proximal tubules of the kidney, seminal vesicle, ciliary body epithelium, hepatocytes, and choroid plexus epithelium. Interestingly, many of these specific epithelia and endothelial cells lining the blood vessels are the tissues in which CD82 is highly expressed. Thus one possible function of CD82 is to regulate removal of oxidized GSH-conjugates.

Oxidative stress has been shown to increase the levels of GGT, as a protective mechanism to maintain high cellular GSH levels and prevent apoptosis [211,213]. In immune cells, the GGT anti-apoptotic

survival mechanism appears to involve activation of NF-kB [214]. Many tumor cells display elevated levels of GGT, which appears to be stimulated by Ras signaling. The elevation in GGT would counteract the extreme oxidative tumor environment and favor tumor cell survival by maintaining a more reducing environment. Elevated levels of GGT would also assist in detoxification of chemotherapeutic drugs. Thus GGT could be a potent tumor survival mechanism in vivo.

Extracellular signal transduction pathways have been shown to locally increase ROS, which is required for cell motility. At the same time these same signaling pathways also protect against high levels of ROS, to prevent cell stress and apoptosis; and do so by induction of new GSH synthesis [215–217]. New GSH synthesis is dependent on influx of the necessary components from outside the cells by GGT. Thus tetraspanin regulation of local GSH levels may be required to maintain cellular homeostasis during normal signaling processes. Whether tetraspanins positively or negatively regulate GGT activity or expression has not been determined. However, based on the observed biological responses, the protective effects conferred by CD53 suggest it would enhance GGT activity. Meanwhile, loss of CD82 – as occurs in metastasis – would appear to favor cell survival and increased motility by allowing more efficient GGT activation. Thus CD82 may normally function to limit GGT activity (Fig. 2B).

Re-expression of CD82 in HT1080 cells was shown to reduce tumor cell survival in the lungs 6 h after tail vein injection [9]. The relatively rapid loss in cell viability, within 6 h of entry into the lungs suggests a very rapid onset of cell death. Alternatively, the cells may have already been induced to die prior to entry into the lungs, during their transit through the blood stream, either through interactions with DARC positive cells or due to loss of cell adhesion. Whatever the mechanism, all these studies strongly suggest that one of CD82's primary functions is to regulate cell survival, be it in the primary tumor, the blood stream, or the colonizing organs. The primary function of CD82 is not to induce cell death per se, since many cell types express CD82 under normal conditions. Rather it may be that CD82 sensitizes cells to extracellular cues that warn the cell that the incorrect microenvironment has been detected, i.e. lack of or incorrect cell–ECM or cell–cell interactions are present. If this is true, then CD82 re-expressing tumor cells ought to be more sensitive to death-inducing agents. In one study stable cell lines expressing CD82 were shown to be more sensitive to apoptosis induced by cycloheximide, but not TNFα [202]. The mechanisms involved in CD82-sensitization needs to be investigated to determine whether any of the cell membrane-interacting partners of CD82 are involved.
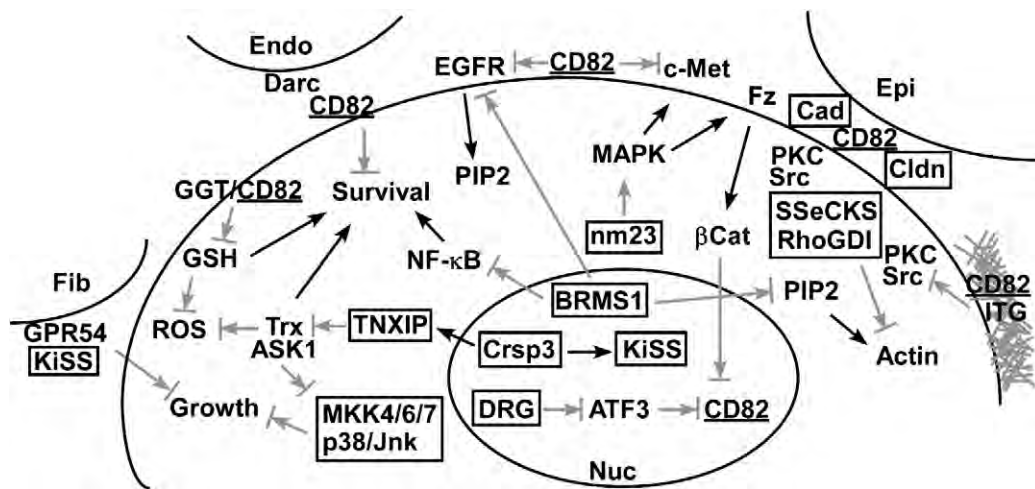
2.10. Relationship of CD82 to other metastasis suppressors

One question that remains to be answered about CD82, and indeed about any metastasis suppressor, is whether loss of a single metastasis suppressor gene in a primary tumor is sufficient for metastasis. Are these genes global regulators to the extent that loss of a single gene would be sufficient to initiate a metastatic cascade in primary tumors? The xenograft models indicate that restoration of a single gene is sufficient to suppress metastasis, suggesting that blocking one step is sufficient to alter the final outcome. This may reflect the complex nature of the metastatic process; many steps are involved and disruption of one is sufficient to abort the process. Alternatively, because of the relationships that may exist between the different suppressors, the activity of one may affect the activity of another. Is there evidence of functional links between the different metastasis suppressors and CD82? Is there redundancy where loss of other suppressors can substitute for loss of CD82? A few potential relationships are presented below and diagrammed in Fig. 3.

2.10.1. Cell–ECM adhesion

nm23 is a kinase that phosphorylates Ksr and reduces MAPK activity. Re-expression of nm23 reduces expression of genes linked to metastasis, such as c-Met, smoothened, frizzled, and MMP2. At least three of these genes, c-Met, frizzled, and MMP2, are potentially impacted by CD82. Two scenarios are possible. Loss of nm23 is sufficient to induce metastasis by simply up regulating c-Met, frizzled, and MMP2 expression. Alternatively, these genes need to be up-regulated, but the suppressive effects of CD82 on their function must also be removed.

KiSS, is a secreted and processed ligand for GPR54 and is a negative regulator of trophoblast migration and invasion [218]. CD82 was

Fig. 3. Molecular relationships between CD82 and other metastasis suppressors. CD82 expression (underline) is potentially controlled by two different suppressors (boxed words). In the nucleus (Nuc) of the tumor cell DRG suppresses the transcription factor ATF3, which negatively regulates *CD82* expression. Enhanced Wnt signaling through frizzled (Fz) to β-catenin (βCat) represses CD82 expression. But Fz expression is in turn suppressed by nm23 via inhibition of MAPK signaling. Nm23 also suppresses c-Met expression. BRMS1 reduces EGFR expression with a concomitant decrease in PIP2 levels, which limits actin reorganization. c-Met and EGFR activation are also suppressed by CD82, which may also contribute to decreased PIP2 levels. CD82 interactions with integrins (ITG) at the matrix, or cadherins (Cad) and claudins (Cldn) at epithelial cell junctions (Epi) stabilizes and limits actin reorganization through suppression of PKC/Src/Rho GTPase signaling which may involve RhoGDI or SSeCKS. CD82 expression increases the sensitivity of cells to death signals through interactions with Darc antigen on endothelial cells (Endo) and suppression of GSH synthesis via GGT. Cell survival signaling is also compromised by BRMS1-mediated reduction in NF-κB signaling. The transcription factor Crsp3 induces two metastasis suppressors, KiSS and TNXIP. TNXIP limits survival via inhibition of the redox protein Trx, but also suppresses cell growth by triggering the MAPK stress response (MKK4/6/7 and p38/Jnk). Simultaneously Crsp3-mediated induction of KiSS and its subsequent binding to GPR54 in surrounding fibroblasts (Fib) triggers a growth suppressive signal to the tumor cell which probably cooperates with the MAPK stress pathway. Thus loss of metastasis suppressors enhances cell signaling leading to enhanced integrin-based motility, loosening of cell–cell junctions, increased cell survival, and ability to grow under stress conditions. All of which are required for successful metastasis.

recently shown to positively control the expression of a protein in decidual cells involved in limiting trophoblast invasion at the maternal–fetal interface [33]. In metastatic cells KiSS binds its receptor on fibroblasts rather than the tumor cells to indirectly suppress tumor cell growth at the site of colonization [219]. CD82 prevents survival of the growth suppressed cells, so KiSS would function downstream of CD82 in a metastatic suppressor cascade.

RhoGDIs sequester Rho GTPases away from their effectors, keeping Rho in an inactive state. RhoGDI2 suppresses metastasis but not tumorigenicity [220]. Active Rho GTPases are critical for cell migration and are regulated by integrin signaling. Direct evidence that CD82 is involved in regulating Rho GTPases in tumor cells has not yet been investigated; however, CD82 and several tetraspanins are known to affect Rho signaling in immune cells. Thus, one possibility is that CD82 facilitates inhibition of Rho GTPases via RhoGDI.

Src-suppressed C kinase substrate (SSeCKS) is a PKC substrate that is negatively regulated by Src. The reported metastasis suppressor functions of SSeCKS strongly resemble those reported for CD82. Re-expression of SSeCKS in tumor cells increases cell–cell adhesion [221], blocks Src-dependent matrigel invasion via inhibition of Rho GTPases [222], and inhibits angiogenesis [223]. The relationship to PKC signaling to SSeCKS and suppression of metastasis has not been investigated. Nonetheless, the strong links between the signaling pathways regulated by CD82 and SSeCKS and the shared biological responses suggests the possibility of interaction between these two metastasis suppressors.

2.10.2. Cell–Cell adhesion

The potential relationships between two metastasis suppressors involved in homotypic cell–cell interactions, Claudin and E-cadherin, were discussed earlier in section 2.7.

2.10.3. Survival

BRMS1 is a transcriptional repressor that suppresses EGFR expression, PIP2 production, and NF-kB transcriptional activity. Its loss promotes adhesion independent survival. It has no impact on the expression of other metastasis suppressors, nm23, CD82, KiSS or E-cadherin. [48,219,224].

The metastasis suppressor Crsp3 is a transcription factor that regulates the expression of two other metastasis suppressors, KiSS and TNXIP. TNXIP/VDUP1 binds the redox-active site of thioredoxin (Trx) to negatively regulate its activity [225]. The Trx system, like the GSH system, acts to reduce intracellular ROS. Trx associates with ASK1, a stress response MAPK kinase. Upon Trx oxidation it dissociates from ASK1 which triggers ASK1 activation and downstream signaling to Jnk/p38 via MKK4/6/7 [226]. Thus loss of TNXIP would enhance the ability of Trx to reduce ROS and maintain cell survival. No links between this pathway and tetraspanins have been reported. The loss of TNXIP may represent an alternative survival mechanism for metastatic cells. Whether there are interactions between Trx and the GSH system with respect to metastasis remains to be established.

DRG1 is nuclear protein whose expression is lost when cells lose PTEN [227]. Re-expression of DRG1 in tumor cells results in a marked decrease in expression of the ATF3 transcription factor. Interestingly, ATF3 binding sites were detected in the CD82 promoter by ChIP analysis (Watabe, personal communication). Tumor samples missing DRG1 also had no CD82, but ATF3 was elevated and vice versa. Thus loss of PTEN, a common occurrence in many tumors and an event associated with progressive disease, would result in loss of DRG1, increased ATF3 and subsequent loss of CD82.

ATF3 is up-regulated in response to stress signaling via Jnk and p38 signaling pathways [228,229]. Stress induced Jnk/p38 signaling due to the tumor microenvironment may also promote ATF3 expression and enhance the loss of CD82. However, elevated ATF3 is also associated with increased apoptosis. Does the loss of CD82 help to diminish the potential apoptotic response?

2.11. Summary

The identification of metastasis suppressor genes and their protein products specifically involved in the complex events associated with the onset and progression of metastatic cancer are providing major insights into the mechanisms of metastasis. The molecular mechanisms by which each of these genes limits metastasis still remain to be fully elucidated. Several specific cellular processes need to be targeted for effective metastasis, and include detachment, motility, invasion, cell survival, and re-growth at the metastatic site. Our current understanding of CD82 function indicates it is likely to be involved in detachment, motility/invasion, and cell survival. As the exact signaling events associated with loss of CD82 and the other suppressor genes become known, as well as the relationship of the signaling pathways to the cellular processes they control, a more comprehensive understanding of which signaling pathways are involved in metastasis becomes evident. With this knowledge comes the ability to design targeted therapies as well as diagnostic and prognostic tests for metastatic cancer.

Acknowledgments

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